

Article

COI Metabarcoding Provides Insights into the Highly Diverse Diet of a Generalist Salamander, *Salamandra salamandra* (Caudata: Salamandridae)

Adam J. D. Marques ^{1,2,3,*} , Vanessa A. Mata ^{1,2} and Guillermo Velo-Antón ^{1,2,4,*} 

¹ CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Campus de Vairão, Universidade do Porto, 4485-661 Vairão, Portugal; vanessamata@cibio.up.pt

² BIOPOLIS Program in Genomics, Biodiversity and Land Planning, CIBIO, Campus de Vairão, 4485-661 Vairão, Portugal

³ Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, 4099-002 Porto, Portugal

⁴ Torre Cacti (Lab 97), Grupo de Ecología Animal, Departamento de Ecología e Biología Animal, Universidade de Vigo, E-36310 Vigo, Spain

* Correspondence: adam.marques@cibio.up.pt (A.J.D.M.); guillermo.velo@uvigo.es (G.V.-A.)

Abstract: DNA metabarcoding has proven to be an accessible, cost-effective, and non-invasive tool for dietary analysis of predators in situ. Although DNA metabarcoding provides numerous benefits in characterizing diet—such as detecting prey animals that are difficult to visually identify—this method has seen limited application in amphibian species. Here, we used DNA metabarcoding to characterize the diet of fire salamanders (*Salamandra salamandra*) (Linnaeus, 1758) in three distinct regions across the northwestern Iberian Peninsula. To test the efficiency of COI-based metabarcoding in determining salamanders' diet diversity, we compared our COI-based results with results from traditional diet studies from neighboring and distant populations, as well as with recent findings obtained in a DNA metabarcoding study using 18S. Two COI primers were used in combination to investigate the potential impact of primer bias in prey detection. Our COI metabarcoding approach increased taxonomic resolution and supported a generalist diet in *S. salamandra*. Between primers, there were no significant differences in the diversity and richness of prey detected. We observed differences in the prevalence of prey identified between sampling regions both in our study and in other studies of *S. salamandra* diet. This COI metabarcoding study provides recommendations and resources for subsequent research using DNA metabarcoding to study amphibian diets.

Keywords: COI; diet; DNA metabarcoding; prey; salamanders



Citation: Marques, A.J.D.; Mata, V.A.; Velo-Antón, G. COI Metabarcoding Provides Insights into the Highly Diverse Diet of a Generalist Salamander, *Salamandra salamandra* (Caudata: Salamandridae). *Diversity* **2022**, *14*, 89. <https://doi.org/10.3390/d14020089>

Academic Editor: Sebastiano Salvadio

Received: 31 December 2021

Accepted: 24 January 2022

Published: 28 January 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Diet studies are fundamental to understanding species' dietary habits [1], food webs [2], and trophic niches [3,4], which are key traits in many ecological processes and for the conservation and management of species and ecosystems. DNA metabarcoding as a means of dietary analysis has been used in many taxonomic groups [5,6], but has been underutilized in amphibians [7]—particularly in salamanders. Salamanders serve an important role as mesofaunal predators [8], often comprising a large portion of ecosystem biomass [9], and have low energy requirements, making them potential energy sinks in ecosystems [10]. Moreover, salamanders may also exert a top-down effect on invertebrate community composition and nutrient cycling [7,11,12], which makes studying the diets of salamander species especially relevant for understanding their role in ecosystem functioning.

Visual inspection of stomach or fecal contents is a useful but inconsistent means of diet characterization in salamanders [13,14]. Stomach contents provide insight into recent consumption [3,15,16], and while stomach-flushing avoids sample mortality, it is an invasive approach to diet analysis [14,16–18]. Fecal content inspection is less invasive, but introduces a bias favoring hard-bodied prey species that are not fully digested [13].

DNA metabarcoding can help to identify prey species that are consumed over a longer period of time, avoids the detection bias against soft-bodied prey, and requires less taxonomic training in prey identification [19]. Similar to visual inspections, DNA metabarcoding can also indicate the preferential diets of salamanders and their role as predators in communities [7,17,20,21], and can inform us about invertebrate biodiversity. To our knowledge, only one study has applied DNA metabarcoding to investigate the diet of adult salamander species. Specifically, Wang et al. [22] used the 18S ribosomal RNA (18S) region to characterize the diets of adult fire salamanders (*Salamandra salamandra*) (Linnaeus, 1758) by collecting fecal samples from three Belgian forests. While 18S has proven useful to detect potential prey items, the use of more informative (i.e., variable) DNA fragments such as the cytochrome c oxidase I (COI) benefits from a large reference database that is supported by the Barcode of Life Data System (BOLD) [23,24], and the relatively high variability of the region allows for high-resolution taxonomic assignment [5,25–27].

This study aims to provide an update on the diet of *S. salamandra* while evaluating the use of COI metabarcoding as an efficient, non-invasive method for diet studies, and comparing it to previous works [15], as well as to gain better insights into the diets and functional roles of salamanders as generalist terrestrial predators [28]. Fecal samples were collected from salamanders across the northwestern Iberian Peninsula. To determine whether a significant difference in prey detection could be attributable to primer bias [29], we compared the performance of two COI primers. Technical considerations include the evaluation of (1) sampling effectiveness to capture prey species, and (2) the usefulness of COI primers as barcodes [7,19,30–33].

2. Materials and Methods

Fecal samples were collected from three distinct regions across the northwestern Iberian Peninsula, including the extended metropolitan area of Porto, a forested area across the Morrazo Peninsula, and the island of Ons, which is mostly dominated by bushes (Figure 1). Nocturnal sampling was conducted in Morrazo and Porto in the spring of 2021, and in Ons during November of 2020, coinciding with the highest annual activity peaks for the species and under suitable climatic conditions (i.e., rainy nights and temperatures of 10–20 °C). Up to 20 individuals from each site were collected and placed in sterilized individual or group containers. All individuals were returned to their original sampling sites.

To mitigate primer bias, two COI-specific primer pairs—fwh1 (fwhF1 5'-YTC HAC WAA YCA YAA RGA YAT YGG-3', fwhR1 5'-ART CAR TTW CCR AAH CCH CC-3') [34] and LerayXT (jgHCO2198 5'-TAI ACY TCI GGR TGI CCR AAR AAY CA-3', 185 bp; mlCOIintF-XT 5'-GGW ACW RGW TGR ACW ITI TAY CCY CC-3'; 313 bp) [27,35]—were used. *Salamandra salamandra* COI sequences from NCBI (KX094979 & GQ380404) were used to design blocking primers for fwh1 (Ssal_fwhF1-blk 5'-CAA AGA CAT TGG CAC CCT CTA CCT AAT TTT TGG [SpC3]-3') and LerayXT (Ssal_mlCOIintF-blk 5'-GAA CAG TCT ACC CCC CCC TTG CCG GAA ATC TGG [SpC3]-3'). Initial PCR mixes comprised 10 µL of Qiagen Multiplex PCR Master Mix, 0.3 µL or 0.4 µL of 10 mM target primer (fwh1 and LerayXT, respectively), 8.0 µL of 10 mM blocking primer, 2 µL of DNA template, and enough water for a final volume of 25 µL [36]. Thermocycling conditions included an initial denaturing step at 95 °C for 15 min, followed by 40 cycles of denaturing at 95 °C for 30 s, annealing at 45 °C for 60 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. Amplification success was verified by running 2 µL of PCR product on a 2% agarose gel. Successfully amplified PCR product was diluted at 1:3 of the initial concentration, in order to reduce the primer dimer during indexing. Illumina indices were then annealed to the PCR product with a PCR composed of 7 µL of KAPA Taq ReadyMix, 1.4 µL of Nextera index [37], 2.8 µL of DNA template, and enough water for a final volume of 14 µL. Thermocycling conditions followed an initial denaturing step at 95 °C for 15 min, followed by 8 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. Indexed PCR products were

purified with AMPure XP beads (Beckman Coulter), eluted to 25 μ L, and pooled into equimolar concentrations per fragment. The pooled libraries were quantified with qPCR, normalized to 4 nM, and sequenced in an Illumina MiSeq with an expected coverage of 20,000 reads per sample.



Figure 1. Maps include the species distribution of *Salamandra salamandra*, our study region in the northwest Iberian Peninsula, and photos illustrating the habitats found within the three sampled regions of Ons, Morrazo, and Porto.

Paired reads were aligned with PEAR [38], and successfully assembled reads went through ‘ngsfilter’ from OBITools [39] to remove primer sequences and annotate sample information. Trimmed reads were then collapsed into unique sequence variants using ‘obiuniq’ and denoised with ‘-cluster-unoise’ from VSEARCH [40], using default parameters, except for minimum sequence length, which was set as 150 bp for fwh1 and 300 bp for LerayXT. Resulting zero-radius operational taxonomic units (zOTUs) went through chimera removal with ‘-uchime3_denovo’, and were clustered at 99% identity [41] with ‘-cluster_size’. Finally, reads were mapped to the remaining OTUs with 99% identity using ‘-usearch_global’. To further remove potential nuclear mitochondrial copies (NUMTs) and surviving PCR and sequencing errors, the R package LULU [42] was used with the default parameters. Extraction and PCR negatives were used to correct for contamination. The maximum number of reads of any OTU identified in either extraction or PCR negative was subtracted from the number of reads observed of that OTU in each sample. OTUs were assigned to a taxon using BOLDIGGER v.1.2.5 [43]. OTUs with a minimum of 90% similarity to a taxon included in the phyla Annelida, Arthropoda, or Mollusca were retained as plausible invertebrate prey [22]. Samples with less than 100 reads assigned to dietary OTUs were discarded, as were OTUs comprising less than 1% of the total dietary reads per sample, so as to avoid errors from tag jumping or overrepresentation of rare prey [44]. Prey items were identified at the genus level, as assignment accuracy at the species level was often missing or undefined in the reference database.

To estimate and compare sampling completeness for each region and fragment, as well as prey richness, we used rarefaction curves based on Hill numbers using the ‘iNEXT’

function from the iNEXT package in R [45,46]. Prey occurrences for each site and for each fragment were converted into incidence frequencies using ‘incfreq’, and then sample coverage and prey richness were calculated. Sample coverage gives us the proportion of the diet composed of prey species already sampled, and is considered a better reference than sample size to compare species richness among differently sampled groups [47]. To compare the prey composition among samples of different regions and fragments, we calculated a pairwise distance matrix using the Jaccard dissimilarity indices using ‘vegdist’ available in the R package vegan [48] to quantify the differences between regions and fragments based on prey occurrence. This matrix was then tested using a permutational multivariate analysis of variance (PERMANOVA) with the Jaccard method and 1000 permutations using ‘adonis’. One of the assumptions of PERMANOVA is that there are no differences in dispersion among groups; thus, we further conducted a beta dispersion test using ‘betadisper’ to confirm this homogeneity. Test results were summarized and displayed via principal coordinates analysis (PCoA). Finally, to assess which prey items were significantly contributing to differences between regions and fragments, we conducted a similarity percentage test using ‘simper’ with 1000 permutations [49].

3. Results

3.1. Sample Collection and Sequence Amplification

A total of 50 individual fecal pellets were extracted (Morrazo = 32, Porto = 8, Ons = 10), with two replicate extractions from a single pellet collected in Porto, and three extraction negatives. Fragments were successfully amplified in 30 samples with fwh1 (Morrazo = 20, Ons = 10) and 38 samples with LerayXT (Morrazo = 21, Porto = 8, Ons = 9), each including the extraction negatives as well as a PCR negative. Post-filtering, we retained dietary reads from a total of 35 individuals, with 25 samples sequenced at either fragment (83% and 66% success rates for fwh1 and LerayXT, respectively) and 15 samples sequenced at both. Each extraction replicate identified three prey taxa, of which two were common to both replicates, while those prey found in only one replicate comprised less than 5% of the total dietary reads.

3.2. Diet Characterization

Across both fragments, a total of 95 unique OTUs were retained, corresponding to 58 prey taxa (Table 1). Two families of Annelida were identified—Almidae and Lumbricidae—wherein one and five genera were identified, respectively. Included among these, *Lumbricus* (present in 49% of samples) was the most common Annelida prey. Arthropoda was the most diverse phylum, comprising 6 known (1 unknown) classes, 18 orders, 32 known (one unknown) families, and 30 known (9 unknown) genera. Among all of these families, no more than two genera were identified. Arthropoda included some of the most common prey—namely, millipedes (Diplopoda), and in particular the genera *Polydesmus* (present among 51% of all samples), *Glomeris* (31%), and *Ommatoiulus* (26%). Mollusca prey comprised only Gastropoda—namely, the orders Pulmonata and Stylommatophora, the former corresponding to a single genus, *Cochlicella*, and the latter comprising 11 families and 12 genera. The second most common prey overall were roundback slugs of the genus *Arion* (49%). While in some instances species-level resolution was available for some prey (e.g., all OTUs assigned to the genus *Glomeris* were also identified as the species *G. occidentalis*), in many cases, taxonomic assignments were unresolved at the species level (e.g., of the nine OTUs assigned to the genus *Arion*, seven different published sequences were identified as matches, but all lacked species-level designations). To avoid potentially inflating the prey richness as an artifact of unresolved taxonomic assignments, we opted instead to use the genus-level resolution, which was available for the majority of OTUs identified.

Table 1. The frequency of occurrence of prey taxa observed among samples in each region, and in total. Where an OTU at the genus-level resolution could not be identified, the next highest taxonomic resolution (e.g., family, order, etc.) is provided. Significant differences in pairwise comparisons of average abundance between regions are shown in bold with an asterisk ($p < 0.05$).

Phylum	Class	Order	Family	Genus	Morrazo	Ons	Porto	Total	
Annelida	Clitellata	Haplotaxida	Almidae	<i>Alma</i>		30 *		9	
			Lumbricidae	<i>Aporrectodea</i>		20		6	
				<i>Dendrobaena</i>	18			9	
				<i>Eisenia</i>	12			6	
				<i>Lumbricus</i>	35	30		26	
				<i>Octolasion</i>	6			3	
Arthropoda	Arachnida	Opiliones	Ischyropsalididae	<i>Ischyropsalis</i>	6			3	
					12			6	
	Trombidiformes	Eupodidae				10		3	
		Cryptopidae	<i>Cryptops</i>	6				3	
	Chilopoda	Scolopendromorpha	Entomobryidae			10		3	
		Collembola	Entomobryomorpha	Isotomidae			10		3
		Poduromorpha				10		3	
						10		3	
						30 *		9	
	Diplopoda	Symphypleona	Hypogastruridae	<i>Hypogastrura</i>		20		6	
			Bourletiellidae			10		3	
		Glomerida	Dicyrtomidae	<i>Dicyrtomina</i>		10		3	
			Sminthurididae			10		3	
			Glomeridae	<i>Glomeris</i>	65			31	
			Julida	Julidae	<i>Cylindroiulus</i>	12		6	
				<i>Ommatoiulus</i>	47		13	26	
		Platydesmida	Andrognathidae		18			9	
	Polydesmida			18			9		
	Insecta	Polydesmida	Paradoxosomatidae	<i>Oxidus</i>			25 *	6	
			Polydesmidae	<i>Polydesmus</i>	88		38	51	
			Cantharidae	<i>Cantharis</i>	12			6	
			Curculionidae	<i>Caenopsis</i>	12			6	
		Coleoptera	Histeridae	<i>Pactolinus</i>		10		3	
			Tenebrionidae	<i>Nalassus</i>	24			11	
			Forficulidae	<i>Forficula</i>		10		3	
			Dolichopodidae	<i>Condyllostylus</i>	12			6	
		Diptera	Ephydriidae	<i>Scatella</i>	12			6	
			Psychodidae	<i>Bichromomyia</i>		10		3	
			Sciaridae	Sciaridae sp.	12			6	
			Sepsidae	<i>Meropliosepsis</i>	6			3	
		Hemiptera	Syrphidae	<i>Eupeodes</i>			13 *	3	
			Aphididae	<i>Chaitophorus</i>			13 *	3	
							25 *	6	
	Malacostraca	Lepidoptera	Noctuidae	<i>Omphaloscelis</i>		10		3	
				<i>Peridroma</i>			13 *	3	
		Orthoptera	Sphingidae	<i>Manduca</i>		10		3	
			Tettigoniidae	<i>Cyrtaspis</i>	6			3	
		Isopoda	Armadillidiidae	<i>Armadillidium</i>		10	38 *	11	
				<i>Eluma</i>	6		63 *	17	
			Oniscidae	<i>Oniscus</i>	6			3	
			Porcellionidae	<i>Porcellio</i>	18			9	
		Mollusca	Gastropoda	Pulmonata	Cochlicellidae	<i>Cochlicella</i>		40 *	
Stylommatophora				Agriolimacidae	<i>Deroceras</i>	12	10	13	11
	Arionidae			<i>Arion</i>	82	30		49	
				<i>Geomalacus</i>	6			3	
	Helicidae			<i>Oestophora</i>		40 *		11	
	Hygromiidae			<i>Portugala</i>			25 *	6	
	Lauriidae			<i>Lauria</i>		10		3	
	Limacidae			<i>Lehmannia</i>		20		6	
	Milacidae			<i>Milax</i>		40 *		11	
	Geomitridae			<i>Ponentina</i>			13	3	
	Oxychilidae			<i>Oxychilus</i>	12			6	
	Testacellidae			<i>Testacella</i>		10		3	
	Urocyclidae			<i>Microkerkus</i>	6			3	

3.3. Species Richness

When comparing samples sequenced at both fragments, we observed a near-identical sample coverage of 65% and 62% for fw1 and LerayXT, respectively, with fw1 detecting a higher number of prey species (39) than LerayXT (25) (Figure 2a). However, when

comparing both fragments at similar levels of sampling completeness, the estimated prey richness did not differ significantly between the two fragments (overlapping 95% confidence intervals of rarefaction curves; Figure 2a). A two-sample *t*-test confirmed that fwh1 produced a higher number of total dietary reads ($10,811 \pm 9896$) post-filtering compared to LerayXT (1802 ± 3015), but no differences in the total number of filtered reads or the ratio of dietary reads to filtered reads ($t = 7.0748$; $p < 0.001$).

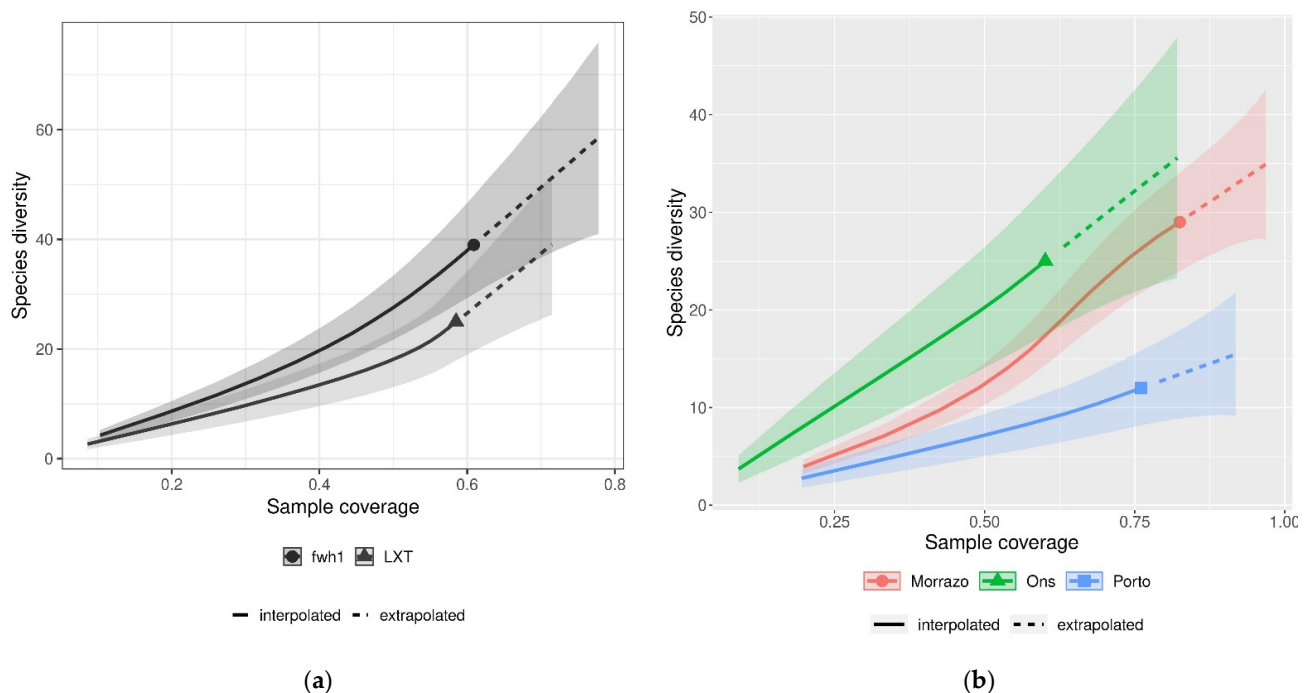


Figure 2. Species diversity extrapolated as a factor of sample coverage for (a) different CO1 fragments; (b) sampling regions. Points indicate the observed richness, while error bars indicate the 95% confidence intervals of the response curves.

Sample coverage was 85%, 72%, and 60% for Morrazo, Porto, and Ons, respectively (Figure 2b). Observed prey richness was 29, 25, and 12 for Morrazo, Ons, and Porto, respectively. Rarefaction curves showed that estimated prey richness in Porto was lower than in Morrazo and Ons, while the latter exhibited similar levels of prey richness. The PERMANOVA model showed no differences in the composition of prey identified by either fragment (Figure 3a), but significant differences between regions ($p < 0.001$). However, the beta dispersal test suggests that the significant differences in prey composition observed in the PERMANOVA between sites may be inflated due to the lack of homogeneity in variance across groups (PCoA; Figure 2b; $p = 0.01967$). Notable differences in prey prevalence between regions include the absence of millipedes among samples from Ons. This coincides with an increased diversity of soft-bodied prey from among Ons samples, including several gastropod genera—*Cochlicella*, *Oestophora*, and *Milax*—found to be significantly more common, and the only instance of earthworms from the family *Alma*. Annelida was notably absent among samples from Porto. Several genera of arthropods, however, were significantly more common in Porto than in other locations, including Polydesmida: *Oxidus*, Diptera: *Eupeodes*, Hemiptera: *Chaitophorus*, Lepidoptera: *Peridroma*, and Isopoda: *Armadillidium* and *Eluma*, although we should note that the low sample coverage from Porto may inflate the significance of this observation.

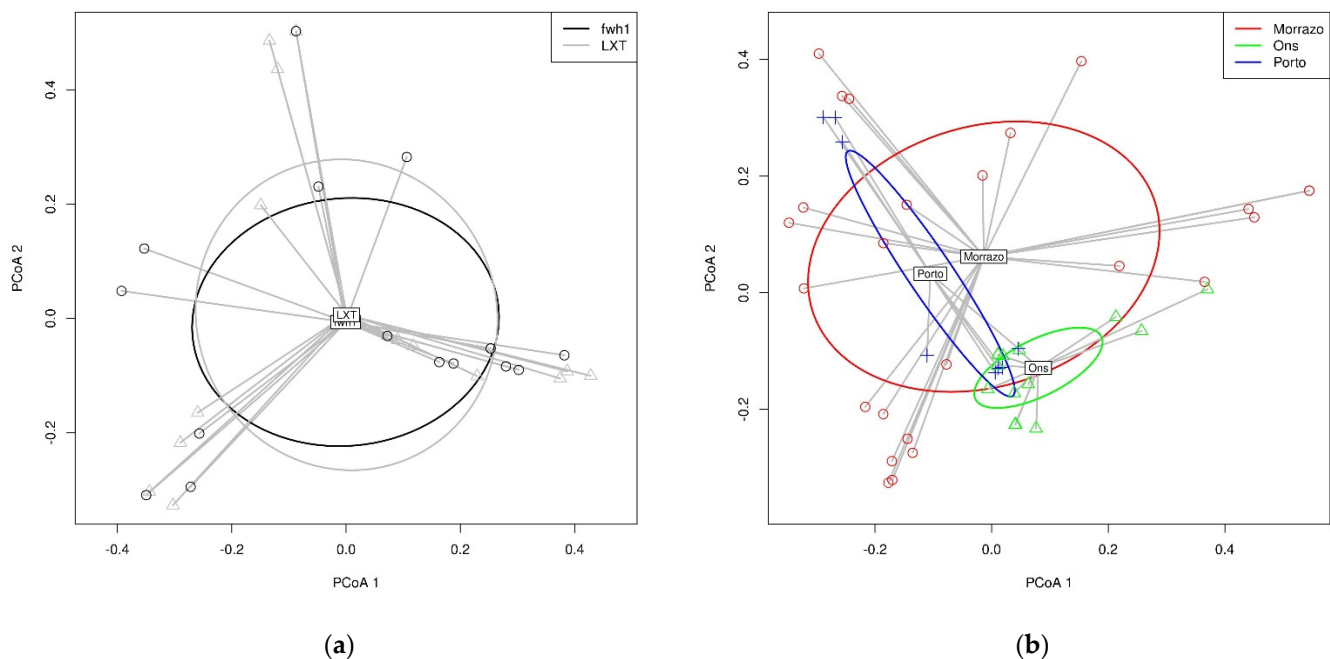


Figure 3. Variance in the prey taxa summarized by the first two eigenvectors of a PCoA for (a) different COI fragments; (b) sampling regions. Ellipses capture the distance of points from the centroid within one standard deviation.

4. Discussion

4.1. COI Metabarcoding for Salamanders' Diet Characterization

Concordant with previous characterizations of *S. salamandra* diet, our results from the DNA metabarcoding of COI suggest a prevalence of low-mobility terrestrial detritivores, including millipedes (Diplopoda), roundback slugs (Arionidae), and earthworms (Lumbricidae) [15–17,50]. While each of these prey taxa are similarly prevalent in the salamander diet as a whole, regional differences such as the absence of Annelids in Porto and Diplopoda in Ons were observed. Comparing the results of COI metabarcoding to diet inspections by Bas et al. [15] in *S. s. gallaica* (northwest Iberia), we observed a clear difference in the types of prey detected. The most common prey identified by Bas et al. [15] were Insecta, with far fewer soft-bodied prey compared to the findings of this study. This was expected, as visual inspection may favor the detection of hard-bodied prey that are more slowly digested compared to soft-bodied prey, which may become visually unrecognizable several days after consumption [13]. We observed this anecdotally in the prevalence of Coleoptera identified by Bas et al. [15], of which 16 genera were identified, as compared to the four genera identified in this study, with both studies identifying *Nalassus* as the most common prey among the class Insecta. Conversely, 18S metabarcoding by Wang et al. [22] found that the most common prey were Gastropoda, which far exceeded other prey in prevalence. Gastropoda have been reported as a common prey elsewhere [16,50], as well as in our results. However, in many of these cases, often only a few prey taxa could be identified, because of either digestion or low taxonomic resolution. Our study identified 58 different prey taxa from three populations at variable taxonomic resolutions—fewer than the 76 prey taxa identified by gastric inspection from a higher number of individuals ($N = 72$), localities ($N = 10$), and a wider environmental and ecological survey [15], but nearly threefold more than from previous taxonomic assignment using DNA metabarcoding of 18S across three forest populations in Belgium [22]. Thus, DNA metabarcoding of COI increases taxonomic resolution and provides a cost-effective and expedient method for characterizing the diets of salamanders. Indeed, the increased taxonomic resolution provided by COI suggests that salamanders are able to utilize a variety of Gastropoda prey. This was most evident among our samples from Ons, which often comprised soft-bodied

gastropods and a complete absence of Diplopoda—possibly as a result of differences in region, season, or habitat compared to our other sites. For instance, seasonal variation was found to influence prey richness in the diets of salamanders, with the greatest prey richness reported in autumn [4,28]. This seems a probable explanation for the observed prey richness among samples from Ons sampled during autumn. Relatively few arachnid (Arachnida) and centipede (Chilopoda) taxa were identified among our samples, although they have been reported in the diets of salamanders [15,22], suggesting an absence either among our samples or in local abundance.

4.2. COI Primers as Barcodes

While studies should always strive to include variable fragments in order to account for primer biases [29,30], our results suggest no discernible difference in the results gathered by using either *fwh1* or *LerayXT*. Greater species richness among *fwh1* sequences compared to *LerayXT* was unexpected given previous comparisons of COI primer performances in literature [51], although sample degradation may favor the shorter fragment. The absence of any clear differences in the prey composition between these primers, however, casts doubt on whether *LerayXT* underperformed, as inequalities in read output and sample coverage discourage definitive conclusions. Further sequencing and more distributive sampling will be necessary for verification. The number of dietary reads generated by *LerayXT* was significantly lower than the number generated by *fwh1*, even with no discernible difference in the prey being identified by either primer. During sequencing, the smaller fragment—in this case, *fwh1*—will usually be favored [52]; however, a comparison between COI primers found that *fwh1* has a higher likelihood of mismatch between the primer and the template, potentially identifying fewer prey species [51]. Despite expectations, *LerayXT* identified fewer prey species, with a possible explanation being among the unfiltered reads, as 32% of all OTUs and 26% of all reads were 352 bp—longer than the target fragment length—and either unassigned or identified as *Flavobacterium*. When present, these OTUs were the most abundant reads among a subset of individuals, and may represent an instance of nuclear mitochondrial DNA (NUMT) resulting from transposition of COI into the nuclear genome [53]. Pseudogenes such as these may evade blocking primers and dominate the amplification reaction. No OTUs were assigned to *Salamandra salamandra*, indicating high efficiency of the blocking primers; however, considering the large size and repetitive nature of the salamander genome, pseudogenes are to be expected [54].

4.3. Dietary Variation across Regions

Although differences in prey prevalence were observed between regions, overlap in the prey composition should deter us from drawing any premature conclusions about diet preferences or prey abundance. Instead, we can refer to these preliminary results as a starting point for subsequent studies. Based on our extended rarefaction results (Supplementary Materials Figure S2), we also recommend that future studies aim for a minimum sample size of 20 sample units per site for sample coverage. The differences in prey taxa observed between Ons and the mainland regions—primarily the prevalence of soft-bodied prey such as land snails and slugs (Stylommatophora) and segmented worms (*Alma*) that were otherwise undetected in the mainland samples—is of particular interest. We might have expected islands to have lower alpha diversity than the mainland [55]; however, samples from Ons were temporally distinct from those of Morrazo and Porto, and must be resampled in the same temporal period in order to control for the known effects that seasonal variation has on prey richness [4,19,50]. Additional observations, such as the relative absence of Diplopoda in the diet of Ons samples, may indicate a scarcity of this common prey, driving prey diversification [56]. In Porto, conversely, we anticipated higher prey richness by taxa—namely, Isopoda and Limacidae—able to utilize anthropogenic spaces [57]. Although prey richness was low when compared to other regions, there was a prevalence of pill woodlice (Armadillidiidae), which may be of interest for studies in ecotoxicology, as terrestrial Isopoda often serve as model organisms in soil

ecotoxicology [58]. However, we must also consider that these samples were not sequenced at fw1, due to poor amplification, and have fewer overall reads to compare ($t = 2.1898$; $p < 0.05$). Previous studies investigating prey consumption in *S. salamandra* detected dietary differences between sexes [22], ages [59], seasons [50], and populations [15]. Follow-up studies should also consider comprehensive sampling of distinct habitats throughout the species' range, as well as the remarkable intraspecific differentiation in reproductive modes [60], head shape [61], and behavioral strategies [62–64], both between and within subspecies of *S. salamandra*. The inclusion of these variables may help to elucidate the factors that contribute to the dietary variation observed in this study.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/d14020089/s1>, Figure S1: Rarefaction comparison between fragments; Figure S2: Rarefaction comparison between regions; Table S1: Extended list of OTUs after sequence annotation and filtration.

Author Contributions: Conceptualization, G.V.-A. and V.A.M.; methodology, V.A.M.; validation, A.J.D.M. and V.A.M.; formal analysis, A.J.D.M.; investigation, G.V.-A. and A.J.D.M.; resources, G.V.-A. and V.A.M.; data curation, G.V.-A., A.J.D.M. and V.A.M.; writing—original draft preparation, A.J.D.M.; writing—review and editing, G.V.-A. and V.A.M.; visualization, A.J.D.M.; supervision, G.V.-A. and V.A.M.; project administration, G.V.-A.; funding acquisition, G.V.-A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Fundação para a Ciência e a Tecnologia, grant numbers PTDC/BIA-EVL/28475/2017 and PTDC/BIA-CBI/2278/2020. V.A.M. was funded by the FCT through the program ‘Stimulus of Scientific Employment, Individual Support—3rd Edition’ (2020.02547.CEECIND). G.V.-A. was supported by the FCT (CEECIND/00937/2018), and recently by a Ramón y Cajal research grant (Ref. RYC-2019-026959-I/AEI/10.13039/501100011033). This work was co-funded by the project NORTE-01-0246-FEDER-000063, supported by Norte Portugal Regional Operational Programme (NORTE2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF).

Institutional Review Board Statement: Fieldwork for collecting individuals and fecal samples was carried out with the corresponding permits from the regional and national administrations (Xunta de Galicia, Ref. EB-031/2021; Portugal Ref. 544/2021/CAPT). Sampling procedures were carried out following the Guidelines for Use of Live Amphibians and Reptiles in Field and Laboratory Research, 2nd Edition, revised by the Herpetological Animal Care and Use Committee (HACC) of the American Society of Ichthyologists and Herpetologists, 2004.

Data Availability Statement: The data presented in this study are openly available in Zenodo at <https://zenodo.org/badge/latestdoi/429739169> (accessed on 30 December 2021).

Acknowledgments: We thank Lucia Alarcón-Ríos and Iria Pazos for assistance during fieldwork, and the staff of the *Parque Nacional Marítimo-Terrestre das Illas Atlánticas de Galicia* for providing lodging and facilitating the trips to the island.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Losos, J.B.; Greene, H.W. Ecological and evolutionary implications of diet in monitor lizards. *Biol. J. Linn. Soc.* **1988**, *35*, 379–407. [CrossRef]
2. Svanbäck, R.; Quevedo, M.; Olsson, J.; Eklöv, P. Individuals in food webs: The relationships between trophic position, omnivory and among-individual diet variation. *Oecologia* **2015**, *178*, 103–114. [CrossRef]
3. Lunghi, E.; Manenti, R.; Cianferoni, F.; Ceccolini, F.; Veith, M.; Corti, C.; Ficetola, G.F.; Mancinelli, G. Interspecific and inter-population variation in individual diet specialization: Do environmental factors have a role? *Ecology* **2020**, *101*, e03088. [CrossRef]
4. Lunghi, E.; Cianferoni, F.; Ceccolini, F.; Veith, M.; Manenti, R.; Mancinelli, G.; Corti, C.; Ficetola, G.F. What shapes the trophic niche of European plethodontid salamanders? *PLoS ONE* **2018**, *13*, e0205672. [CrossRef]
5. De Sousa, L.L.; Silva, S.M.; Xavier, R. DNA metabarcoding in diet studies: Unveiling ecological aspects in aquatic and terrestrial ecosystems. *Environ. DNA* **2019**, *1*, 199–214. [CrossRef]
6. Ando, H.; Mukai, H.; Komura, T.; Dewi, T.; Ando, M.; Isagi, Y. Methodological trends and perspectives of animal dietary studies by noninvasive fecal DNA metabarcoding. *Environ. DNA* **2020**, *2*, 391–406. [CrossRef]

7. Ficetola, G.F.; Manenti, R.; Taberlet, P. Environmental DNA and metabarcoding for the study of amphibians and reptiles: Species distribution, the microbiome, and much more. *Amphibia-Reptilia* **2019**, *40*, 129–148. [\[CrossRef\]](#)
8. Laking, A.E.; Li, Z.; Goossens, E.; Miñarro, M.; Beukema, W.; Lens, L.; Bonte, D.; Verheyen, K.; Pasmans, F.; Martel, A. Salamander loss alters litter decomposition dynamics. *Sci. Total Environ.* **2021**, *776*, 145994. [\[CrossRef\]](#)
9. Peterman, W.E.; Crawford, J.A.; Semlitsch, R.D. Productivity and significance of headwater streams: Population structure and biomass of the black-bellied salamander (*Desmognathus quadramaculatus*). *Freshw. Biol.* **2007**, *53*, 347–357. [\[CrossRef\]](#)
10. Davic, R.D.; Welsh Jr, H.H. On the ecological roles of salamanders. *Annu. Rev. Ecol. Evol. Syst.* **2004**, *35*, 405–434. [\[CrossRef\]](#)
11. Walton, B.M. Salamanders in forest-floor food webs: Environmental heterogeneity affects the strength of top-down effects. *Pedobiologia* **2005**, *49*, 381–393. [\[CrossRef\]](#)
12. Hocking, D.J.; Babbitt, K.J. Effects of red-backed salamanders on ecosystem functions. *PLoS ONE* **2014**, *9*, e86854. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Costa, A.; Salvidio, S.; Posillico, M.; Altea, T.; Matteucci, G.; Romano, A. What goes in does not come out: Different non-lethal dietary methods give contradictory interpretations of prey selectivity in amphibians. *Amphibia-Reptilia* **2014**, *35*, 255–262. [\[CrossRef\]](#)
14. Crovetto, F.; Romano, A.; Salvidio, S. Comparison of two non-lethal methods for dietary studies in terrestrial salamanders. *Wildl. Res.* **2012**, *39*, 266–270. [\[CrossRef\]](#)
15. Bas Lopez, S.; Gutian Rivera, J.; Castro Lorenzo, A.d.; Sanchez Canals, J.L. Data on the diet of salamander (*Salamandra salamandra* L.) in Galicia. *Bol. Estac. Cent. Ecol.* **1979**, *8*, 73–78.
16. Çiçek, K.; Koyun, M.; Tok, C.V. Food composition of the Near Eastern Fire Salamander, *Salamandra infraimmaculata* Martens, 1885 (Amphibia: Urodela: Salamandridae) from Eastern Anatolia. *Zool. Middle East* **2017**, *63*, 130–135. [\[CrossRef\]](#)
17. Solé, M.; Rödder, D. Dietary assessments of adult amphibians. In *Amphibian Ecology and Conservation: A Handbook of Techniques*; Dodd, C.K., Jr., Ed.; Oxford University Press: Oxford, UK, 2009; pp. 167–184.
18. Salvidio, S.; Pasmans, F.; Bogaerts, S.; Martel, A.; van de Loo, M.; Romano, A. Consistency in trophic strategies between populations of the Sardinian endemic salamander *Speleomantes imperialis*. *Anim. Biol.* **2017**, *67*, 1–16. [\[CrossRef\]](#)
19. Alberdi, A.; Aizpurua, O.; Bohmann, K.; Gopalakrishnan, S.; Lynggaard, C.; Nielsen, M.; Gilbert, M.T.P. Promises and pitfalls of using high-throughput sequencing for diet analysis. *Mol. Ecol. Resour.* **2019**, *19*, 327–348. [\[CrossRef\]](#)
20. Unger, S.D.; Williams, L.A.; Diaz, L.; Jachowski, C.B. DNA barcoding to assess diet of larval eastern hellbenders in North Carolina. *Food Webs* **2020**, *22*, e00134. [\[CrossRef\]](#)
21. Unger, S.D. Adult female Eastern hellbender *Cryptobranchus alleganiensis* (Cryptobranchidae) conspecific cannibalism confirmed via DNA barcoding. *Herpetol. Notes* **2020**, *13*, 169–170.
22. Wang, Y.; Smith, H.K.; Goossens, E.; Hertzog, L.; Bletz, M.C.; Bonte, D.; Verheyen, K.; Lens, L.; Vences, M.; Pasmans, F.; et al. Diet diversity and environment determine the intestinal microbiome and bacterial pathogen load of fire salamanders. *Sci. Rep.* **2021**, *11*, 20493. [\[CrossRef\]](#)
23. Hebert, P.D.N.; Ratnasingham, S.; de Waard, J.R. Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proc. R. Soc. Lond. B* **2003**, *270*, S96–S99. [\[CrossRef\]](#)
24. Ratnasingham, S.; Herbert, P.D.N. BOLD: The Barcode of Life Data System (<http://www.barcodinglife.org>). *Mol. Ecol. Notes* **2007**, *7*, 355–364. [\[CrossRef\]](#)
25. Borrell, Y.J.; Miralles, L.; Do Huu, H.; Mohammed-Geba, K.; Garcia-Vazquez, E. DNA in a bottle—Rapid metabarcoding survey for early alerts of invasive species in ports. *PLoS ONE* **2017**, *12*, e0183347. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Grey, E.K.; Bernatchez, L.; Cassey, P.; Deiner, K.; Deveney, M.; Howland, K.L.; Lacoursière-Roussel, A.; Leong, S.C.Y.; Li, Y.; Olds, B.; et al. Effects of sampling effort on biodiversity patterns estimated from environmental DNA metabarcoding surveys. *Sci. Rep.* **2018**, *8*, 8843. [\[CrossRef\]](#)
27. Wangenstein, O.S.; Palacín, C.; Guardiola, M.; Turon, X. DNA metabarcoding of littoral hard-bottom communities: High diversity and database gaps revealed by two molecular markers. *PeerJ* **2018**, *6*, e4705. [\[CrossRef\]](#)
28. Velo-Antón, G.; Buckley, D. Salamandra común—*Salamandra salamandra*. In *Enciclopedia Virtual de los Vertebrados Españoles*; Salvador, A., Martínez-Solano, I., Eds.; Museo Nacional de Ciencias Naturales: Madrid, Spain, 2015; Available online: www.vertebradosibericos.org/ (accessed on 22 November 2021).
29. Pereira, A.; Samlali, M.A.; S'Khifa, A.; Slimani, T.; Harris, D.J. A pilot study on the use of DNA metabarcoding for diet analysis in a montane amphibian population from North Africa. *Afric. J. Herpetol.* **2021**, *70*, 68–74. [\[CrossRef\]](#)
30. Zinger, L.; Bonin, A.; Alsos, I.G.; Bálint, M.; Bik, H.; Boyer, F.; Chariton, A.A.; Creer, S.; Coissac, E.; Deagle, B.E.; et al. DNA metabarcoding—Need for robust experimental design to draw sound ecological conclusions. *Mol. Ecol.* **2019**, *28*, 1857–1862. [\[CrossRef\]](#) [\[PubMed\]](#)
31. Clarke, L.J.; Soubrier, J.; Weyrich, L.S.; Cooper, A. Environmental metabarcodes for insects: In silico PCR reveals potential for taxonomic bias. *Mol. Ecol. Resour.* **2014**, *14*, 1160–1170. [\[CrossRef\]](#)
32. Deagle, B.E.; Jarman, S.N.; Coissac, E.; Pompanon, F.; Taberlet, P. DNA metabarcoding and the cytochrome c oxidase subunit I marker: Not a perfect match. *Biol. Lett.* **2014**, *10*, 20140562. [\[CrossRef\]](#)
33. da Silva, L.P.; Mata, V.A.; Lopes, P.B.; Lopes, R.J.; Beja, P. High-resolution multi-marker DNA metabarcoding reveals sexual dietary differentiation in a bird with minor dimorphism. *Ecol. Evol.* **2020**, *10*, 10364–10373. [\[CrossRef\]](#)

34. Vamos, E.; Elbrecht, V.; Leese, F. Short COI markers for freshwater macroinvertebrate metabarcoding. *PeerJ Prepr.* **2017**, *1*, e14625. [CrossRef]
35. Geller, J.; Meyer, C.; Parker, M.; Hawk, H. Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Mol. Ecol. Resour.* **2013**, *13*, 851–861. [CrossRef]
36. Lopes, R.J.; Pinho, C.J.; Santos, B.; Seguro, M.; Mata, V.A.; Egeter, B.; Vasconcelos, R. Intricate trophic links between threatened vertebrates confined to a small island in the Atlantic Ocean. *Ecol. Evol.* **2019**, *9*, 4994–5002. [CrossRef] [PubMed]
37. Adey, A.; Morrison, H.G.; Asan, X.; Kitman, J.O.; Turner, E.H.; Stackhouse, B.; MacKenzie, A.P.; Caruccio, N.C.; Zhang, X.; et al. Rapid, low-input, low-bias construction of shotgun fragment libraries by high-density in vitro transposition. *Genome Biol.* **2010**, *11*, R119. [CrossRef] [PubMed]
38. Zhang, J.; Kobert, K.; Flouri, T.; Stamatakis, A. PEAR: A fast and accurate Illumina Paired-End reAd merger. *Bioinformatics* **2014**, *30*, 614–620. [CrossRef]
39. Boyer, F.; Mercier, C.; Bonin, A.; Le Bras, Y.; Taberlet, P.; Coissac, E. obitools: A unix-inspired software package for DNA metabarcoding. *Mol. Ecol. Resour.* **2016**, *16*, 176–182. [CrossRef] [PubMed]
40. Rognes, T.; Flouri, T.; Nichols, B.; Quince, C.; Mahé, F. VSEARCH: A versatile open source tool for metagenomics. *PeerJ* **2016**, *4*, e2584. [CrossRef] [PubMed]
41. Mata, V.A.; Ferreira, S.; Campos, R.M.; da Silva, L.P.; Veríssimo, J.; Corley, M.F.V.; Beja, P. Efficient assessment of nocturnal flying insect communities by combining automatic light traps and DNA metabarcoding. *Environ. DNA* **2020**, *3*, 398–408. [CrossRef]
42. Frøslev, T.G.; Kjeller, R.; Bruun, H.H.; Ejrnæs, R.; Brunbjerg, A.K.; Pietroni, C.; Hansen, A.J. Algorithm for post-clustering curation of DNA amplicon data yields reliable biodiversity estimates. *Nat. Commun.* **2017**, *8*, 1188. [CrossRef]
43. Buchner, D.; Leese, F. BOLDigger—A Python package to identify and organise sequences with the Barcode of Life Data systems. *Metabarcoding Metagenom.* **2020**, *4*, 19–21. [CrossRef]
44. Deagle, B.E.; Thomas, A.C.; McInnes, J.C.; Clarke, L.J.; Vesterinen, E.J.; Clare, E.L.; Kartzinel, T.R.; Paige, E. Counting with DNA in metabarcoding studies: How should we convert sequence reads to dietary data? *Mol. Ecol.* **2019**, *28*, 391–406. [CrossRef] [PubMed]
45. Hsieh, T.C.; Ma, K.H.; Chao, A. iNEXT: An R package for rarefaction and extrapolation of species diversity (Hill numbers). *Methods Ecol. Evol.* **2016**, *7*, 1451–1456. [CrossRef]
46. Chao, A.; Gotelli, N.J.; Hsieh, T.C.; Sander, E.L.; Ma, K.H.; Colwell, R.K.; Ellison, A.M. Rarefaction and extrapolation with Hill, numbers: A framework for sampling and estimation in species diversity studies. *Ecol. Monogr.* **2014**, *84*, 45–67. [CrossRef]
47. Chao, A.; Jost, L. Coverage-based rarefaction and extrapolation: Standardizing samples by completeness rather than size. *Ecology* **2012**, *93*, 2533–2547. [CrossRef]
48. Oksanen, J.; Blanchet, F.G.; Friendly, M.; Kindt, R.; Legendre, P.; McGinn, D.; Minchin, P.R.; O'Hara, R.B.; Simpson, G.L.; Solymos, P.; et al. Vegan: Community Ecology Package. R Package Version 2.5-7. 2020. Available online: <https://CRAN.R-project.org/package=vegan> (accessed on 30 December 2021).
49. Clarke, K.R. Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol.* **1993**, *18*, 117–143. [CrossRef]
50. Balogová, M.; Miková, E.; Orendáš, P.; Uhrin, M. Trophic spectrum of adult *Salamandra salamandra* in the Carpathians with the first note on food intake by the species during winter. *Herpetol. Notes* **2015**, *8*, 371–377.
51. Piñol, J.; Senar, M.A.; Symondson, W.O.C. The choice of universal primers and the characteristics of the species mixture determine when DNA metabarcoding can be quantitative. *Mol. Ecol.* **2019**, *28*, 407–419. [CrossRef]
52. Taberlet, P.; Bonin, A.; Zinger, L.; Coissac, E. DNA sequencing. In *Environmental DNA: For Biodiversity Research and Monitoring*; Oxford University Press: Oxford, UK, 2018. [CrossRef]
53. Andújar, C.; Creedy, T.J.; Arribas, P.; López, H.; Salces-Castellano, A.; Pérez-Delgado, A.J.; Vogler, A.P.; Emerson, B.C. Validated removal of nuclear pseudogenes and sequencing artefacts from mitochondrial metabarcoding data. *Mol. Ecol. Resour.* **2021**, *21*, 1772–1787. [CrossRef]
54. Weisrock, D.W.; Hime, P.M.; Nunziata, S.O.; Jones, K.S.; Murphy, M.O.; Hotelling, S.; Kratochvil, J.D. Surmounting the Large-Genome “Problem” for Genomic Data Generation in Salamanders. In *Population Genomics: Wildlife*; Hohenlohe, P.A., Rajora, O.P., Eds.; Springer International Publishing AG: Cham, Switzerland, 2018. [CrossRef]
55. Russell, J.C.; Kueffer, C. Island Biodiversity in the Anthropocene. *Annu. Rev. Environ. Resour.* **2019**, *44*, 31–60. [CrossRef]
56. Steinmetz, R.; Seuaturien, N.; Intanajitjuy, P.; Inrueang, P.; Prempre, K. The effects of prey depletion on dietary niches of sympatric apex predators in Southeast Asia. *Integr. Zool.* **2021**, *16*, 19–32. [CrossRef] [PubMed]
57. Ferenti, S.; David, A.; Nagy, D. Feeding-behaviour responses to anthropogenic factors on *Salamandra salamandra* (Amphibia, Caudata). *Biharean Biol.* **2010**, *4*, 139–143.
58. Van Gestel, C.; Loureiro, S.; Idar, P. Terrestrial isopods as model organisms in soil ecotoxicology: A review. *ZooKeys* **2018**, *801*, 127–162. [CrossRef] [PubMed]
59. Kuzmin, S.L. Feeding ecology of *Salamandra* and *Mertensiella*: A review of data and ontogenetic evolutionary trends. *Mertensiella* **1994**, *4*, 271–286.
60. Mulder, K.P.; Alarcón-Ríos, L.; Nicieza, A.G.; Fleischer, R.C.; Bell, R.C.; Velo-Antón, G. Independent evolutionary transitions to pueriparity across multiple timescales in the viviparous genus *Salamandra*. *Mol. Phylogenet. Evol.* **2022**, *167*, 107347. [CrossRef]

-
61. Alarcón-Ríos, L.; Nicieza, A.G.; Kaliontzopoulou, A.; Buckley, D.; Velo-Antón, G. Evolutionary history and not heterochronic modifications associated with viviparity drive head shape differentiation in a reproductive polymorphic species, *Salamandra salamandra*. *Evol. Biol.* **2020**, *47*, 43–55. [[CrossRef](#)]
 62. Steinfartz, S.; Weitere, M.; Tautz, D. Tracing the first step to speciation: Ecological and genetic differentiation of a salamander population in a small forest. *Mol. Ecol.* **2007**, *16*, 4550–4561. [[CrossRef](#)]
 63. Manenti, R.; Denoël, M.; Ficetola, G.F. Foraging plasticity favours adaptation to new habitats in fire salamanders. *Anim. Behav.* **2013**, *86*, 375–382. [[CrossRef](#)]
 64. Velo-Antón, G.; Cordero-Rivera, A. Ethological and phenotypic divergence in insular fire salamanders: Diurnal activity mediated by predation? *Acta Ethol.* **2017**, *20*, 243–253. [[CrossRef](#)]