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DNA metabarcoding analysis of stomach flushing contents reveals the exceptionally diverse diet of the golden alpine salamander

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Amphibians, particularly salamanders, are facing dramatic population declines worldwide; therefore, investigating their role as predators through diet analysis could inform targeted conservation programs. DNA metabarcoding significantly outperforms morphology-based identification of prey taxa in other vertebrates, but has rarely been applied to amphibians, and then only for faecal samples. Here, we chose the golden Alpine salamander *Salamandra atra aurorae*, an endangered endemism of the Italian Alps, to (i) develop a new metabarcoding protocol for amphibian stomach flushing samples; (ii) investigate the diet of this subspecies, including differences in the Frequency of Occurrence (%FO) of prey taxa between sexes; and (iii) compare these results with a recently published morphology-based identification of diet for the same samples. From 53 golden Alpine salamanders from the Province of Trento (Italy) population, targeting two COI fragments with DNA metabarcoding, we detected an extraordinary 177 prey taxa (103 to species), obtaining a much higher proportion of positive samples and taxonomic resolution compared to the morphological study. The FO of these taxa showed a strong positive correlation between sexes. In amphibian diet studies, the inclusion of metabarcoding protocols for stomach flushes could accelerate our understanding of their trophic ecology and improve future conservation actions.

Keywords DNA metabarcoding, Diet, Golden Alpine salamander, *Salamandra atra aurorae*, Stomach flushing, Invertebrate biodiversity

According to the latest Global Amphibian Assessment[1], amphibians are the most threatened Vertebrate Class globally due to the spread of new pathogens, the climate crisis, habitat loss, and overexploitation. In particular, the conservation status of salamanders is worsening rapidly worldwide, as the Red List Index of the Order Caudata is consistently the lowest[1]. In this context, studies focused on their feeding behavior and preferred prey have significantly advanced the understanding of trophic interactions[2], ecosystem network dynamics[3], predator-prey dynamics[4], and the overall impact of species on their environment[5]. In terms of habitat conservation, diet studies help to identify critical foraging areas and preferred food sources[6]. This information is essential for the development of targeted conservation plans aimed at preserving ecosystems and mitigating the threats posed by habitat loss, degradation, and overexploitation[7], as well as the presence of parasitic[8] and invasive species[9,10].

The study of the amphibian diet is traditionally based on morphological identification of their prey from stomach flushing samples, which is considered a minimally invasive, non-lethal method in amphibians (e.g.[11,12]). Compared to fecal samples, which are often the preferred sample type for other species as they can be collected without disturbing or harming individuals (e.g., lizards:[13]; felids:[14]), stomach flushes appear to provide more reliable diet composition than faeces in the case of amphibians[15,16].

Morphological-based identification of taxa detected in both stomach flush and faecal samples, however, has its own limitations when compared to molecular approaches such as DNA metabarcoding. For instance, DNA metabarcoding protocols of these sample types can identify species even when morphologically diagnostic

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characters are absent or unrecognizable due to partial or complete digestion[17]. If comprehensive DNA barcodes reference databases are available, prey identification with this method could potentially reach higher taxonomic resolution with respect to the morphological approach[17]. In fact, numerous studies on non-amphibian species have confirmed that DNA metabarcoding has significantly greater resolution and better accuracy than morphological identification (e.g., Natterer's bat *Myotis nattereri*[18]; European hake *Merluccius merluccius*[7]; Selvagens gecko *Tarentola (boettgeri) bischoffi*[19]; Blue Catfish *Ictalurus furcatus*[20]). This is especially the case for samples from animals with generalist diets, such as some insectivores[21]. Very few DNA metabarcoding-based studies of amphibian diet have been published thus far, and all of these use faecal samples[8,22–24]. To the best of our knowledge, this is the first study to use DNA metabarcoding to analyze amphibian stomach flushing samples.

Therefore, here we aimed to (i) develop a new DNA metabarcoding protocol for the analysis of stomach flushing contents in amphibians; (ii) apply this protocol to the golden Alpine salamander *Salamandra atra aurorae* (Trevisan, 1982) as a case study; (iii) compare the efficiency of our new protocol with available morphological identification[25], in order to highlight advantages and drawbacks of each method. This work aimed at providing useful information for conservation planning of the target taxon by investigating its dietary habits, including potential sex differences.

Methods

Study species and study area

The golden Alpine salamander (*S. a. aurorae*), a subspecies of the Alpine salamander *S. atra* (Laurenti, 1768), is endemic to the Italian Alps with a very limited distribution of only 26 km². Two main populations are known, located on the Vezzena Plateau in the Autonomous Province of Trento (Trentino-Alto Adige/Südtirol Region) and the Sette Comuni Plateau in the neighboring Province of Vicenza (Veneto Region;[26]). In the latest IUCN Red List assessment for Italy (2022), the conservation status of this subspecies was reclassified from Vulnerable (VU) to Endangered (EN), due to habitat degradation and its declining distribution and occupancy area[27].

The sampling area for this study was located in the Municipality of Levico Terme on the Vezzena Plateau (Fig. 1). This plateau has a mean elevation of 1450 m a.s.l. and a typically Alpine climate, but with humidity levels similar to the neighboring pre-Alpine Veneto Region[28]. The vegetation consists mainly of silver fir (*Abies alba* Mill., 1759), beech (*Fagus sylvatica* L., 1753), and spruce (*Picea abies* (L.) H. Karst., 1881[29]). Of note, on 29 October 2018, the 'Vaia' windstorm felled millions of trees in the northeastern Alps, including a large number in the study area, causing significant damage to the forest and possibly threatening this species' survival[25].

Sample collection

The sampling design and protocol are detailed in[25]. Briefly, the study area was divided into 29 square plots (20 × 20 m), which were surveyed for adult salamanders on the 26, 28 and 29 July 2022 (during or immediately after rainfall). All experimental procedures were approved by the Italian Ministry of Ecological Transition (authorization MiTE-0014200 of the 7 February 2022) and were performed in accordance with relevant guidelines and regulations. Sampling was performed as follows: 53 *S. a. aurorae* adult individuals (defined as total length > 90 mm;[30]) were captured in the wild by hand using sterile gloves, placed in a sterile container, and

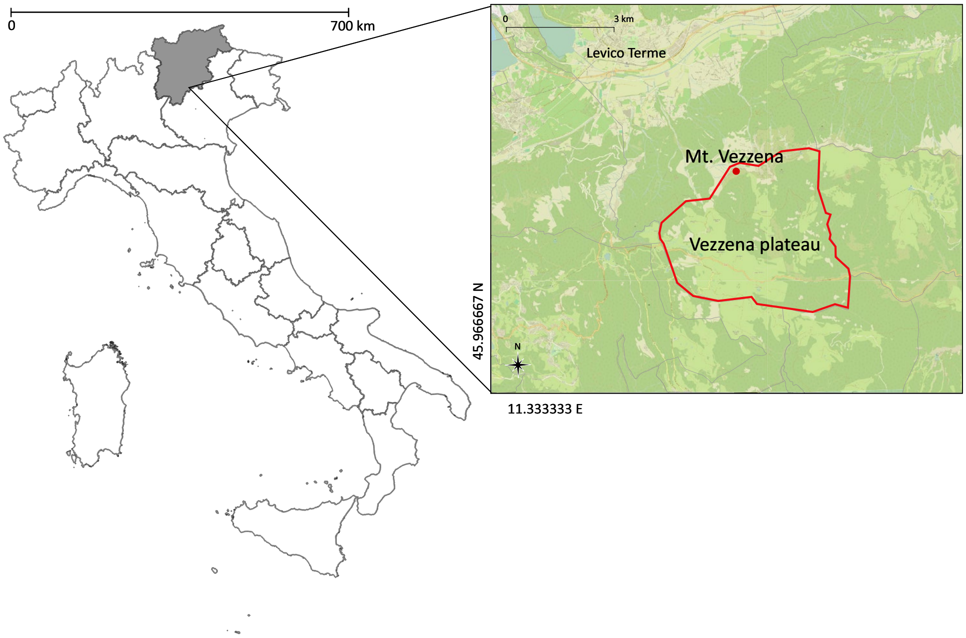

Fig. 1. Map of the general study area, with the Vezzena Plateau (Trentino-Alto Adige/Südtirol Region, Italy) outlined in red. Image created with QGIS.org (2024) QGIS Geographic Information System. Open Source Geospatial Foundation Project http://qgis.org.

kept at 4 °C to reduce metabolic rate and slow down digestion[31]. Stomach flushing samples were obtained using 10–30 ml water to flush the stomach into sterile 50 ml Falcon tubes using the protocol established by Fraser[32]. Sex and reproductive state were noted for each individual. Nine of the 53 samples did not contain visible prey remains. No mortality or stress reactions were observed from the animals during the entire sampling process. Samples were kept at room temperature in 70% ethanol until further analysis, while sampled individuals were released in their respective capture plots within 6 h after capture.

Sample processing

First, morphological analysis was carried out by MUSE - Science Museum of Trento, and results were published in[25]. To minimize sample DNA contamination at this step, researchers wore sterile gloves (changed between each sample) and worked with sterile Petri dishes and forceps. Then, after morphological identification, stomach contents and liquid (see below) were immediately transferred to new sterile Falcon tubes and transferred at room temperature to the Animal, Environmental and Antique DNA Platform of the Conservation Genomics Research Unit (FEM, S. Michele all'Adige, Italy), where metabarcoding analysis was carried out.

Since each sample contained up to 100 ml of 70% ethanol (added during the stomach flushing process), in order to concentrate the DNA, before DNA extraction, each sample was mechanically fractured and homogenized in liquid nitrogen using a sterile ceramic pestle and mortar, and the homogenate was filtered through a Sterivex-GP 0.22 µm Filter unit (Millipore, Burlington, Massachusetts, USA). All the following procedures were performed under BSL2 biological hoods. Total DNA extraction was carried out from the filters using the DNeasy PowerWater Sterivex Kit (Qiagen®), following manufacturer's instructions except for modifications noted in[33]. At least one negative control was included in each extraction batch. Extracted DNA was eluted in 100µL EB buffer.

PCR amplification targeted the Cytochrome Oxidase I (COI) gene, which is commonly used for DNA metabarcoding of invertebrates (e.g.[24,34–36]). In order to broaden the taxonomic scope of this study, two primer pairs were used here, each including standard Illumina sequencing adapters: ZBJ-ArtF1c and ZBJ-ArtR2c (hereafter PRIMER_A) targeting a 157 bp (bp) fragment[37], and mlCOIintF-XT with jgHCO2198, as modified by Wangensteen et al.[38], targeting a 313 bp sequence (here, PRIMER_B). PCR reaction mix for PRIMER_A consisted of 10 µl of Promega Flexi Buffer 5X, 6 µl of $MgCl_2$ 25µM, 2 µl of each forward and reverse primers 10ρmol/µl, 0.25 µl of dNTP's 10mM each, 0.25 µl of Promega - GoTaq HS G2 5U/µL, 5 µl of template DNA and sterile H_2O up to a volume of 50 µl. The reaction mix for PRIMER_B was the same, except that 1 µl of dNTP's 10mM each. The PCR reaction programs for both primers are detailed in Figure S1 (Supplementary Information). One PCR negative control (reaction mix only), as well as the extraction negative controls, were included for each PCR reaction.

After amplification success was confirmed on a QIAxcel Advanced System (QIAGEN, Germany), all samples were purified with the MinElute PCR Purification Kit (QIAGEN, Germany). Library preparation followed guidelines of the Nextera XT Index Kit (FC-131-1001/FC-131-1002; Illumina, Inc., San Diego, California, USA). All samples were quantified and normalized, before being pooled together to a final concentration of 13ρM. Libraries were then sequenced on an Illumina MiSeq platform with the 2 × 300 bp paired-end approach and a sequencing depth of 30,000 reads per sample.

Data analysis

Bioinformatic analysis was carried out with MICCA[39]. Briefly, raw paired-end reads were merged with a minimum overlap length of 50 bp, and a maximum of 10 mismatches. After primer trimming, the sequences were filtered by quality and length allowing an expected error rate of 0.1%, and minimum lengths of 155 bp for PRIMER_A and 300 bp for PRIMER_B. Sequences were dereplicated, denoised, and filtered for any PCR/sequencing errors or chimeras with the *denovo_unoise* MICCA command, which implements the UNOISE3 algorithm[40], to obtain Amplicon Sequence Variants (ASVs). Finally, to eliminate potential contamination from the dataset, the corresponding number of reads of each ASV found in either extraction or PCR negative controls was subtracted from each sample[24]. Singletons were also removed. Details on the commands and parameters used for this bioinformatic analysis are shown in Figure S2 (Supplementary Information).

Since a reference database of COI sequences for local invertebrate fauna was not available, ASVs detected by both PRIMER_A and PRIMER_B were identified with BLAST[41], only considering the top result (i.e. the results with lowest e-value). ASVs were considered assigned only if they met the following criteria: (i) they belonged to the Phyla Annelida, Arthropoda, or Mollusca; (ii) they shared at least 99% of coverage with the matching GenBank reference; and (iii) they passed the minimum identity thresholds of 90% for family, 95% for genus, and 98% for species. Any unassigned ASV was discarded. To further validate these results, the plausibility that each species could be found in the sampling area was checked with presence records from the Global Biodiversity Information Facility (GBIF; https://www.gbif.org/) and the Checklist of Species of the Italian Fauna (https://www.faunaitalia.it/checklist/). In addition, the ecological role of each taxon was noted after consulting expert taxonomists at FEM and peer-reviewed literature[42–44].

The dietary preference of *S. a. aurorae* was quantified by calculating the percent Frequency of Occurrence (%FO) for each prey taxon as the percentage of samples in which the taxon was detected out of the total number of samples, considering the combined results obtained with both primer pairs[45,46]. The correlation between %FO of the main prey taxa found in the stomach of males (34 individuals) versus females (19), and of pregnant (11) versus non-pregnant (8) females, was tested with the Spearman's rank correlation coefficient in RStudio using the cor.test function[47]. Scatterplots of these correlations were produced with *ggplot2*[48] and edited with Inkscape[49]. %FOs, represented with barplots, was also used to qualitatively compare results between metabarcoding analysis (this study) and morphology-based identifications (published in[25]).

Results

All 53 stomach flushing samples were successfully amplified on the Qiaxcel Screening System and, therefore, were sequenced together with the negative controls, obtaining a total of 32,761,400 raw reads. Following bioinformatic merging and denoising, the total dataset consisted of 3,199,813 reads, including 1,233,061 reads of PRIMER_A and 1,966,752 of PRIMER_B. Overall, 320 ASVs were identified for PRIMER_A and 1,267 for PRIMER_B. Of these ASVs, 201 for PRIMER_A and 618 for PRIMER_B were taxonomically classified with BLAST. Our DNA metabarcoding protocol was able to identify from 2 to 29 prey taxa in all 53 samples (mean: 9.4 ± 2.0 taxa). Overall, 177 taxa, including 103 species, were identified; 52 (29.4%) were detected by both primers, 65 (36.7%) only by PRIMER_A, and 60 (33.9%) only by PRIMER_B (details shown in Table S1, Supplementary Materials).

The most common taxa in the diet of *S. a. aurorae* were Diptera (%FO: 84.9%), Myriapoda (76%), Arachnida (77.4%; including Aranea with %FO: 58.5%, Opiliones 35.8%, and Acarina 5.7%), Coleoptera (56.6%), and Psocoptera (50.9%), followed by Collembola (45.3%), Lepidoptera (39.6%), Clitellata (34%), Hemiptera (24.5%), and Gastropoda (22.6%). The least common were Neuroptera (13.2%), Dermaptera (3.8%), and Malacostraca (1.9%). The complete list of taxa detected with DNA metabarcoding in this study and %FO for each are shown in Table 1. Of the 177 identified taxa, six species (the house fly *Helina troene* Walker, 1849; the Highlands thricops *Thricops albibasalis* Zetterstedt, 1849; the parasitoid wasps *Dusona angustifrons* Forster, 1868, *Enizemum ornatum* Gravenhorst, 1829, and *Syrphoctonus longiventris* Thomson, 1890; and the booklouse *Stenopsocus lachlani* Kolbe, 1880), four genera (*Leptomias*, *Muscopterix*, *Metylophorus* and *Amphigerontia*), and one family (Pemphigidae) had a known distribution limited to the Americas, Asia, or northern Europe, and were therefore not expected to be found in our study area (Table 1).

The proportion of taxa detected showed a very strong positive correlation between the diet of male and female salamanders (Spearman's rho: 0.923; p-value < 0.001; Fig. 2A). The proportion of prey found in samples also showed a positive correlation between females with different reproductive status (pregnant or non-pregnant; Spearman's rho: 0.797; p-value < 0.001; Fig. 2B).

Using the same samples as in Centomo et al.[25], our metabarcoding protocol identified at least one prey item in a higher number of samples compared to the morphological analysis (53 here, versus 44 as reported in[25]), and more total taxa (177 versus 16 in[25]), and achieved a higher taxonomic resolution (103 species here, compared to 16 higher-level taxa, Family at best, in[25])and Diptera; Neuroptera and Psocoptera. In addition, of the taxa detected by both methods, metabarcoding showed a higher frequency of occurrence (%FO), meaning that when a taxon was detected it was generally found in a higher proportion of samples with respect to the morphological study (as shown in Fig. 3), with two exceptions (Acarina and Isopoda). On the other hand, DNA metabarcoding failed to detect two taxa which were found by Centomo et al.[25], albeit at low %FO, namely Anoplura, represented in Fig. 3, and Pseudoscorpiones, included in the Arachnida Class for morphological data in Fig. 3.

Discussion

As amphibians are rapidly declining worldwide, studies investigating their feeding behavior and diet preferences are fundamental to understanding trophic interactions, ecosystem network and predator-prey dynamics, in addition to identifying critical foraging areas and preferred food sources (e.g.[4]). More in general, this information is fundamental for a better understanding of the biology and behavior of these elusive species[17] and has great conservation implications in terms of explaining populations fluctuations, the impact of habitat degradation and the climate crisis, or for establishing successful Conservation Breeding Programs (CBP)[50]. Although our target species is not involved in any CBP at present, these programs are, more in general, becoming increasingly relevant to support amphibian conservation in light of their ongoing population decline[51].

The DNA metabarcoding protocol developed here aimed, for the first time, at rapidly identifying prey taxa from stomach flushing samples, a minimally invasive sampling method for amphibians. This approach obtained a detailed list of prey from all samples collected during this study, and the comparison of these results with a traditional taxonomic study of the same samples proved our DNA metabarcoding protocol to be a fast and reliable alternative, although a few taxon-specific limitations still exist. The application of this method provided insights into the diet of the endangered subspecies used here as a case study, the golden Alpine salamander *S. a. aurorae*, which was shown to ingest a remarkable 177 taxa, 103 of which we were able to identify to species. As salamanders are currently threatened worldwide, the development of minimally invasive protocols such as the one presented here is fundamental for comprehensive conservation-oriented programs to identify the resources required by target species. The application of metabarcoding to *S. a. aurorae* stomach flushes offers valuable ecological insights that can inform conservation strategies. As an endangered taxon with a restricted distribution, understanding its trophic interactions is crucial for habitat management and protection. Our findings indicate that *S. a. aurorae* consumes a diverse range of invertebrates, reflecting a flexible feeding strategy. This adaptability may provide resilience in the face of certain environmental changes, but it also underscores the importance of maintaining prey biodiversity within its habitat, especially that of soil-dwelling invertebrates. The main threats to *S. a. aurorae* include illegal collection for pet trade, habitat alteration due to forestry practices, and potential climate-driven changes in microhabitat conditions[52]. A precise identification of the taxonomic composition of the golden Alpine salamander diet, as provided by our study, can establish an important baseline for monitoring shifts in trophic ecology and for assessing the compatibility of land use practices with foraging requirements. Furthermore, the notable presence, in the salamander's diet, of taxa associated with dead wood on the forest floor[53] underscores the importance of appropriate forest management, as previously highlighted[25,54]. In addition, diet studies of generalist species such as S. *a. aurorae* potentially provide information on smaller, more elusive invertebrates, shedding light on their diversity within forest ecosystems and helping to assess habitat integrity[55]. Therefore, our approach should be extended to other amphibian species and habitats. The knowledge gained from this research, being relevant for both predator diet and soil invertebrate biodiversity, could assist in the

Class	%FO	Order	%FO	Family	%FO	Genus	%FO	Species	%FO
Phylum: Annelida	**34.0**								
Clitellata	**34.0**	**Arhynchobdellida**	**28.3**	Xerobdellidae	28.3	*Xerobdella*	28.3		
		Crassiclitellata	**9.4**	Lumbricidae	9.4	*Bimastos*	7.5	*Bimastos rubidus*	7.5
						Octolasion	1.9	*Octolasion lacteum*	1.9
Phylum: Arthropoda	**100.0**								
Arachnida	**77.4**	**Araneae**	**58.5**	Agelenidae	17.0	*Histopona*	9.4	*Histopona torpida*	9.4
				Amaurobiidae	24.5	*Amaurobius*	1.9	*Amaurobius ruffoi*	1.9
						Callobius	22.6	*Callobius claustrarius*	22.6
				Cybaeidae	7.5	*Cybaeus*	7.5	*Cybaeus tetricus*	7.5
				Linyphiidae	41.5	*Agyneta*	1.9	*Agyneta rurestris*	1.9
						Labulla	1.9	*Labulla thoracica*	1.9
						Neriene	1.9	*Neriene peltata*	1.9
						Pityohyphantes	7.5	*Pityohyphantes phrygianus*	7.5
						Tenuiphantes	32.1	*Tenuiphantes alacris*	5.7
								Tenuiphantes tenebricola	30.2
		Opiliones	**35.8**	Lacinius	7.5	*Lacinius*	7.5		
				Nemastomatidae	1.9	*Mitostoma*	1.9	*Mitostoma chrysomelas*	1.9
				Phalangiidae	18.9	*Lophopilio*	9.4	*Lophopilio palpinalis*	9.4
						Mitopus	17.0	*Mitopus morio*	6.5
				Trogulidae	7.5	*Trogulus*	3.8		
		Sarcoptiformes	**3.8**	Acaridae	1.9	*Tyrophagus*	1.9	*Tyrophagus putrescentiae*	1.9
				Scheloribatidae	1.9	*Scheloribates*	1.9	*Scheloribates initialis*	1.9
		Trombidiformes	**3.8**	Eupodidae	3.8				
Chilopoda	**9.4**	**Lithobiomorpha**	**9.4**	Lithobiidae	9.4	*Lithobius*	1.9	*Lithobius tenebrosus*	1.9
Collembola	**45.3**	**Entomobryomorpha**	**45.3**	Entomobryidae	1.9				
				Isotomidae	1.9				
				Tomoceridae	43.4				
Diplopoda	**71.7**	**Chordeumatida**	**37.7**	Chordeumatidae	7.5	*Chordeuma*	7.5	*Chordeuma sylvestre*	7.5
				Neoatractosomatidae	35.8	*Pseudocraspedosoma*	35.8	*Pseudocraspedosoma grypischium*	35.8
		Glomerida	**28.3**	Glomeridae	28.3	*Glomeris*	22.6	*Glomeris klugii*	18.9
								Glomeris undulata	17.0
						Onychoglomeris	9.4	*Onychoglomeris tyrolensis*	9.4
		Polydesmida	**41.5**	Polydesmidae	41.5	*Polydesmus*	41.5	*Polydesmus complanatus*	5.7
								Polydesmus monticola	7.5
		Julida	**1.9**	Julidae	1.9				
Insecta	**100.0**	**Coleoptera**	**56.6**	Alexiidae	1.9	*Sphaerosoma*	1.9		
				Cantharidae	3.8				
				Carabidae	**24.5**	*Carabus*	11.3	*Carabus creutzeri*	11.3
						Pterostichus	13.2	*Pterostichus burmeisteri*	1.9
								Pterostichus unctulatus	11.3
				Coccinellidae	1.9	*Aphidecta*	1.9	*Aphidecta obliterata*	1.9
				Curculionidae	24.5	*Dryocoetes*	15.1	*Dryocoetes autographus*	15.1
						Hylastes	1.9	*Hylastes cunicularius*	1.9
						Hylurgops	1.9	*Hylurgops palliatus*	1.9
						Ips	1.9	*Ips typographus*	1.9
						*Leptomias**	5.7		
				Elateridae	9.4	*Ampedus*	9.4	*Ampedus nigrinus*	9.4
				Melyridae	3.8	*Dasytes*	3.8	*Dasytes cyaneus*	3.8
				Ptinidae	1.9	*Episernus*	1.9	*Episernus granulatus*	1.9
				Zopheridae	5.7	*Coxelus*	5.7	*Coxelus pictus*	5.7
		Dermaptera	**3.8**	Forficulidae	3.8	*Chelidura*	3.8	*Chelidura acanthopygia*	3.8
		Diptera	**84.9**	Anthomyiidae	13.2	*Hylemya*	7.5	*Hylemya nigrimana*	7.5
								Hylemya vagans	13.2
						Lasiomma	7.5		
				Cecidomyiidae	26.4	*Didactylomyia*	3.8	*Didactylomyia longimana*	3.8
				Empididae	5.7	*Hilara*	5.7	*Hilara litorea*	5.7

Continued

Class	%FO	Order	%FO	Family	%FO	Genus	%FO	Species	%FO
				Ephydridae	5.7				
				Keroplatidae	3.8	*Macrocera*	3.8	*Macrocera parva*	3.8
				Limoniidae	20.8	*Metalimnobia*	20.8	*Metalimnobia quadrinotata*	1.9
								Metalimnobia solitaria	9.4
								Metalimnobia tenua	20.8
				Muscidae	32.1	*Coenosia*	7.5	*Coenosia mollicula*	3.8
						Helina	5.7	*Helina fraterculа*	1.9
								Helina obtusipennis	5.7
								*Helina troene**	1.9
						Limnophora	5.7		
						Thricops	20.8	*Thricops albibasalis**	20.8
								Thricops rufisquamus	7.5
				Mycetophilidae	3.8				
				Phoridae	3.8	*Megaselia*	3.8	*Megaselia errata*	3.8
				Rhagionidae	3.8	*Rhagio*	3.8	*Rhagio lineola*	3.8
				Scathophagidae	5.7	*Norellia*	5.7	*Norellia tipularia*	5.7
				Sciaridae	20.8	*Scatopsciara*	3.8	*Scatopsciara atomaria*	3.8
				Syrphidae	35.8	*Baccha*	1.9	*Baccha elongata*	1.9
						Dasysyrphus	7.5	*Dasysyrphus lenensis*	7.5
						Meliscaeva	5.7	*Meliscaeva cinctella*	5.7
						Parasyrphus	11.3	*Parasyrphus lineolus*	3.8
								Parasyrphus macularis	7.5
						Syrphus	17.0	*Syrphus torvus*	15.1
								Syrphus vitripennis	1.9
				Tachinidae	11.3	*Admontia*	1.9	*Admontia grandicornis*	1.9
						*Muscopteryx**	5.7		
						Trichactia	1.9	*Trichactia pictiventris*	1.9
						Voria	1.9	*Voria ruralis*	1.9
				Tipulidae	7.5	*Tipula*	7.5	*Tipula hortorum*	5.7
								Tipula nubeculosa	1.9
								Tipula truncorum	3.8
		Hemiptera	**24.5**	Aphididae	24.5	*Cinara*	22.6	*Cinara confinis*	3.8
								Cinara pectinatae	18.9
						Phyllaphis	5.7	*Phyllaphis fagi*	5.7
				Pemphigidae*	1.9				
		Hymenoptera	**45.3**	Athaliidae	1.9	*Athalia*	1.9	*Athalia circularis*	1.9
				Braconidae	13.2	*Homolobus*	1.9		
						Pauesia	11.3		
				Chrysididae	1.9	*Pseudomalus*	1.9	*Pseudomalus violaceus*	1.9
				Eucharitidae	1.9				
				Ichneumonidae	7.5	*Dusona*	1.9	*Dusona angustifrons**	1.9
						Enizemum	1.9	*Enizemum ornatum**	1.9
						Syrphoctonus	1.9	*Syrphoctonus longiventris**	1.9
						Woldstedtius	1.9	*Woldstedtius biguttatus*	1.9
								Woldstedtius holarcticus	1.9
				Tenthredinidae	24.5	*Tenthredo*	24.5	*Tenthredo atra*	20.8
		Lepidoptera	**39.6**	Erebidae	1.9	*Atolmis*	1.9	*Atolmis rubricollis*	1.9
				Gelechiidae	3.8	*Eulamprotes*	3.8	*Eulamprotes libertinella*	3.8
				Geometridae	5.7	*Eupithecia*	5.7	*Eupithecia tantillaria*	3.8
								Eupithecia vulgata	1.9
				Noctuidae	30.2	*Autographa*	1.9	*Autographa gamma*	1.9
						Euxoa	9.4	*Euxoa nigricans*	9.4
						Mythimna	26.4	*Mythimna ferrago*	26.4
				Pyralidae	1.9	*Plodia*	1.9	*Plodia interpunctella*	1.9
				Tortricidae	11.3	*Celypha*	9.4	*Celypha lacunana*	9.4
						Epinotia	1.9	*Epinotia nana*	1.9

Continued

Class	%FO	Order	%FO	Family	%FO	Genus	%FO	Species	%FO
		Mecoptera	**1.9**	Panorpidae	1.9	*Panorpa*	1.9	*Panorpa germanica*	1.9
		Neuroptera	**13.2**	Chrysopidae	3.8	*Nineta*	3.8	*Nineta vittata*	3.8
				Hemerobiidae	9.4	*Hemerobius*	9.4	*Hemerobius atrifrons*	1.9
								Hemerobius micans	3.8
								Hemerobius pini	3.8
		Psocoptera	**50.9**	Liposcelididae	1.9	*Liposcelis*	1.9	*Liposcelis corrodens*	1.9
				Peripsocidae	34.0	*Peripsocus*	34.0	*Peripsocus subfasciatus*	34.0
				Psocidae	22.6	*Amphigerontia**	1.9		
						*Metylophorus**	7.5		
						Psococerastis	9.4	*Psococerastis gibbosa*	9.4
				Stenopsocidae	1.9	*Stenopsocus*	1.9	*Stenopsocus lachlani**	1.9
Malacostraca	**1.9**	**Isopoda**	**1.9**	Trachelipodidae	1.9	Trachelipus	1.9	*Trachelipus ratzeburgii*	1.9
Phylum: Mollusca	**22.6**								
Gastropoda	**22.6**	**Stylommatophora**	**22.6**	Arionidae	5.7	Arion	3.8	*Arion alpinus*	1.9
				Clausiliidae	3.8	Macrogastra	3.8		
				Helicidae	1.9	Causa	1.9	*Causa holosericea*	1.9
				Hygromiidae	5.7				
				Vitrinidae	5.7	Vitrinobrachium	5.7		

Table 1. Frequency of occurrence (%FO) of prey taxa detected with DNA metabarcoding of the stomach flushing samples of *Salamandra atra aurorae* individuals from the Province of Trento (Italy). Where a species-level identification was not available, the lowest possible taxonomic level is provided. Taxa also found in[25] are highlighted in bold, while taxa with distributions unknown from the study area are marked with *.

rapid development of integrated conservation and management strategies, which are essential for the protection of the world's most threatened vertebrate group and their habitats.

Overall, our results align with previous research on other *Salamandra* species[8,24], which predominantly prey on Diptera, Myriapoda, Aranea, Coleoptera, and Psocoptera. However, with 177 taxa detected, including 103 species, the golden Alpine salamander's diet appears to be richer with respect to the fire salamander *S. salamandra*, for which Marques et al.[24] found 58 prey taxa (48 genera), and Wang et al.[8] 20 prey taxa. Even lower numbers were detected in three sympatric *Desmognathus* species in another recent metabarcoding study (11 genera or species for *D. ocoee*, 11 for *D. amphileucus*, and 22 for *D. monticola*[23]). However, it should be noted that these discrepancies might be due to the different sample type used in these previous studies (faecal samples) and/or to marker choice (18S[8]; COI, but different single set of primers[23]). In fact, the effect of prey digestion on morphological identification of prey items in amphibian species has been reported before[15,16]. As for DNA metabarcoding studies, stomach content samples are known to contain better quality DNA compared to fecal samples[56], although to the best of our knowledge no studies comparing DNA metabarcoding data from stomach flushes and fecal samples in amphibians have been published so far.

Based on our results, a strong correlation was observed between the dietary composition of both males and females, suggesting that *S. a. aurorae* does not exhibit strong sexual dietary dimorphism and has a generalist trophic strategy. Similarly, no substantial dietary divergence was detected between pregnant and non-pregnant females, indicating that reproductive status does not significantly influence prey selection in this taxon.

Although promising, the DNA metabarcoding approach still presents technical challenges. For instance, the need for using multiple primer pairs in DNA metabarcoding diet studies is currently debated. Although it has been argued that choosing appropriate primers according to each study design can effectively eliminate the need for multiple primer sets, at least in studies focusing on insect species (e.g., insect mock community[57]), combinations of different primers are still used where the expected targets encompass a broader taxonomic range (e.g., diet of three horseshoe bats[58]; of three insectivore mammals[59]; of *S. salamandra*[24]). In this study, which targeted Annelida and Mollusca in addition to Arthropoda, 29.4% of the 177 taxa identified were detected by both primers, 36.7% only by PRIMER_A, and 33.9% only by PRIMER_B. Therefore, the use of two primers greatly improved the overall dataset, and we conclude that a combination of primer sets should be used when studying the diet of generalist species like the golden Alpine salamander.

Overall, our results confirm the potential of metabarcoding studies to reach a much higher taxonomic resolution and accuracy with respect to morphological analysis (e.g.[7,60]). The comparison of results from the DNA metabarcoding protocol presented here and those from the morphological analysis published in Centomo et al.[25] showed that the former performed better in terms of number of positive samples, number of total taxa identified, taxonomic resolution, and %FO obtained, for taxa that were found by both methods. The false negatives detected in the DNA metabarcoding approach (i.e., Pseudoscorpiones and Anoplura, which were found by[25] but not in the present study) may be attributed to the lack of comprehensive reference COI sequences for these taxa (e.g.[61]); to differential amplification biases of the primers chosen[59]; or DNA degradation of certain prey items. However, as the two missing taxa were found in less than 5% of the samples in[25], our overall knowledge of the golden Alpine salamander's diet was not changed significantly by using a molecular approach. Importantly, however,

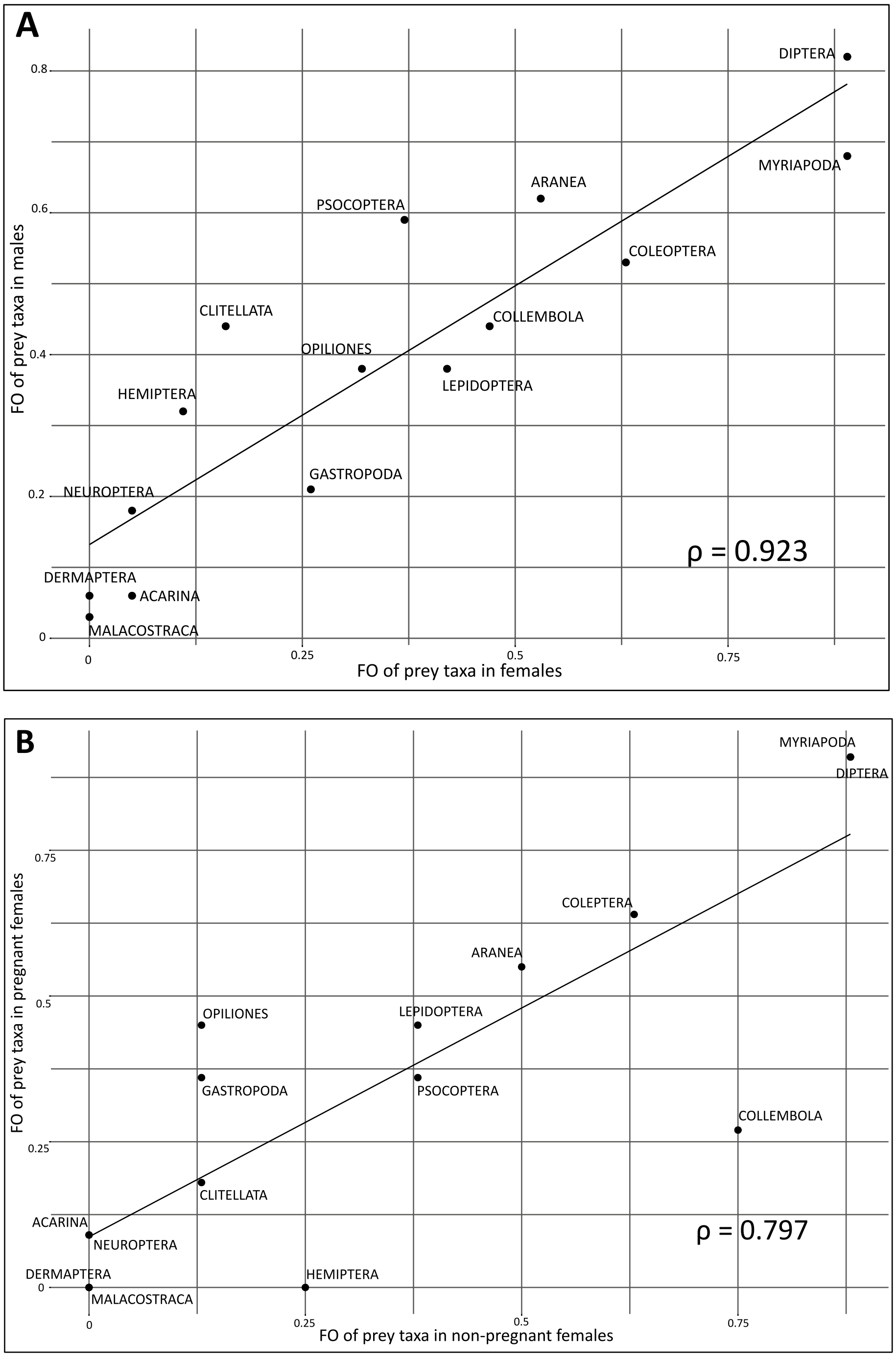

Fig. 2. Scatterplots showing the correlations between the Frequency of Occurrence (FO) of each prey taxon in *Salamandra atra aurorae* (**A**) male and female diet: x-axis: females; y-axis: males, and (**B**) pregnant and non-pregnant female diets: x-axis: non-pregnant females; y-axis: pregnant females. Black lines represent the correlation trend line, and Spearman's rho values are also reported. Plots created with the R package *ggplot2* and edited with Inkscape.

it should be noted that DNA metabarcoding cannot at present distinguish between prey life stages, providing less ecological information than morphology-based identifications[62,63]. Furthermore, metabarcoding does not provide other data, such as the abundance of each prey taxon[64], which is relevant to some aspects of trophic ecology such as trophic strategy, prey selectivity and inter-individual diet variation. Given the limitations of both methods, we conclude that an integrated approach represents the most effective way to investigate the diet of amphibians.

The detection of a few taxa with geographical distributions inconsistent with our study area suggests that some sequences may have originated from contaminated reference databases or sequencing errors, a challenge

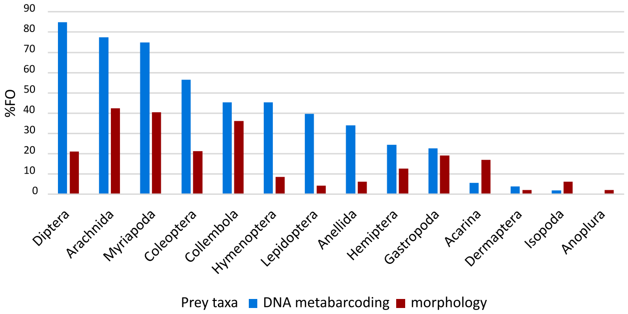

Fig. 3. Comparison of the Frequency of Occurrence (%FO) of each prey taxon found by DNA metabarcoding (blue; data from this study) or morphology (dark red; data from[25]) in *Salamandra atra aurorae* stomach flushing samples.

inherent to DNA metabarcoding studies. According to GBIF, however, two species and two genera found here have been detected in areas geographically close to the study area (i.e., central Italy, eastern France, Austria, and southern Germany: *E. ornatum;* Austria: *S. lachlani*; northern Italy and in the Veneto Prealps: *Metylophorus;* southern Europe: *Amphigerontia*). Additionally, it should be noted that Pemphigidae are currently considered a subfamily under the name Eriosomatinae[65,66], and the latter has also been recorded in northern Italy and Slovenia. The identification of the remaining taxa by metabarcoding could be explained as misassignments, due to undetected PCR/sequencing errors, or to errors or biases linked to the reference database[67–69]. Nonetheless, considering the current rate of alien species translocation, further investigation into the invertebrate fauna of the study area would help to confirm, or rule out, the presence of these 'unlikely' taxa.

Most prey taxa found in the stomach flushing samples are known to dwell in the soil litter for at least one life stage, and could therefore plausibly become prey of the target species[42–44,70]. However, as some of the taxa detected are known parasites or predators (i.e., Hymenoptera: Braconidae, Ichneumonidae, and Chrysididae parasitize Lepidoptera and Diptera; Neuroptera and Psocoptera prey on aphids[43]), part of the biodiversity identified by metabarcoding could be secondary prey. While this possibility needs to be considered when drawing conclusions on the target species' ecology and behavior, nonetheless the DNA metabarcoding approach represents a powerful survey methodology to investigate the complexity of the trophic network of ground dwelling animals. In this scenario, combining morphological and metabarcoding approaches could help distinguish between primary and secondary prey.

While our study confirms the advantages of DNA metabarcoding in dietary studies, certain methodological challenges must also be acknowledged. The use of COI primers, although effective for a wide range of invertebrates, introduces inherent biases due to differential amplification efficiencies across taxa: some prey groups may be underrepresented or entirely missed if they possess a lower primer affinity[59]. Future studies could mitigate this limitation by incorporating additional genetic markers or employing shotgun sequencing approaches to increase taxonomic coverage. Furthermore, the reliability of prey identification is contingent upon the completeness of reference databases[61]. Our manual verification of species distributions helped reduce potential misidentifications, but continued efforts to expand DNA barcode libraries for regional invertebrate fauna would further enhance accuracy.

Data availability

The dataset generated and analyzed in this study is publicly accessible on the EMBL Database, Project Accession: PRJEB86487 (https://www.ebi.ac.uk/ena/browser/view/ERP169832).

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Author contributions

Conceived and designed the study: all authors; acquired the funding: M.G., A.R., P.P., H.C.H.; collected the samples: F.P., L.R., A.R.; designed the molecular protocol: L.Z, F.P., M.G., H.C.H.; performed laboratory analyses: F.P., M.G.; completed bioinformatic and statistical analyses: L.Z., F.P.; drafted the manuscript: L.Z., H.C.H; read, commented and approved the manuscript: all authors.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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