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Research Article

Otter diet and prey selection in a recently recolonized area assessed using microscope analysis and DNA barcoding

Fabio Marcolin[☆1,2,*], Francesca Iordan[☆2], Elisabetta Pizzul[3], Alberto Pallavicini[3], Valentina Torboli[3], Chiara Manfrin[3], Lorenzo Quaglietta[4]

[1] *Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa.*
[2] *Therion Research Group*
[3] *Department of Life Sciences, University of Trieste*
[4] *CIBIO/InBio – Centro de Investigação em Biodiversidade e Recursos Genéticos da Universidade do Porto*

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Abstract

Ecologists traditionally investigate the feeding ecology of vertebrates by visual identification of prey in their scats using a microscope. This analysis, however, presents some pitfalls, such as poor prey identification at genus/species level. DNA barcoding is an alternative to the traditional diet analysis, recently applied to investigate the diet of several vertebrates. It allows the identification of cryptic or not-recognizable prey portions basing their identification on the analysis of conserved DNA fragments. Here, we combined microscope analysis with DNA barcoding to investigate the diet of the Eurasian otter (*Lutra lutra*) in an area recently recolonized by the species, in the surroundings of Tarvisio, NE Italy. We collected 102 spraints and analysed all of them at microscope. A random subsample (N=50) was then analysed through DNA barcoding, via amplification of the V9 region of the SSU 18S-rDNA 18S gene. Furthermore, we assessed seasonal variation in otter diet and whether otters select certain salmonids size classes, to identify potential conflict with anglers. According to the microscope analysis, otters ate primarily fish (salmonids and European bullhead), secondarily amphibians and, more rarely, crayfish, both annually and seasonally. DNA barcoding analysis confirmed the microscope results, attributing some salmonids sequences to the brown trout (*Salmo trutta*). The lack of potential prey species reference sequences in the GenBank database hampered us in identifying the other taxa found in the spraints at more informative levels. Consumed salmonids and bullheads were of small size. In particular, otters selected salmonids size class of 100 mm, which is much smaller than the minimum catchable size for angling. Overall, our study confirms DNA barcoding potential for otter diet analyses, highlighting the need of additional markers to improve molecular prey identification and suggesting that otters do not constitute a significant threat to anglers in the area.

Introduction

Dietary studies are a major tool to unravel the trophic processes undergoing in biological communities, providing valuable information to understand the ecology, evolution and conservation of animals forming those communities (Kartzinel et al., 2015; Symondson, 2002). In particular, the traditional analysis of faecal samples at microscope is one of the more ancient, and yet more widespread, method to assess the diet of carnivores, as these are most-often secretive and thus their diet cannot be easily studied through direct observation (Klare et al., 2011). Traditional microscope analysis, however, has several limitations, including difficulties in detecting some taxa (Krawczyk et al., 2016; Klare et al., 2011; Braley et al., 2010).

To overcome such limitations, several authors in recent years have used the DNA barcoding method to examine the diet of vertebrates (Smith et al., 2018; Biffi et al., 2017; Mata et al., 2016; Santos et al., 2015; Shehzad et al., 2012; Deagle et al., 2009). Several studies on the diet of carnivores, which used both traditional and genetic analysis, reported better prey identification accuracy with the latter (Santos et al., 2015; Shehzad et al., 2012; Deagle et al., 2009). DNA barcoding method, however, is limited when quantitative data (e.g. size class of prey) are needed (Pompanon et al., 2012; Shehzad et al., 2012; Zeale et al., 2011; King et al., 2008; Valentini et al., 2009) and calibration protocol and system are still under debate/development (Deagle et al., 2019). Thus, cryptic, and particularly threatened, species may still widely benefit from a combined approach using both morphological (traditional) analysis, to obtain quantitative information, and DNA-based analysis, to increase species identification rate (Barnett et al., 2010). The diet of the Eurasian otter (*Lutra lutra* L., 1758) has been widely studied in most of its range, being traditionally assessed by microscope inspection of 'spraints' (otter scats) (Kruuk, 2006). Spraints are indeed easy to find, otter preys are easy to identify through a microscope and the method is cheap (Kruuk 2006). Missing detections and under or over estimation of the importance of some prey, however, are known to negatively affect the traditional analysis of otter diet (Krawczyk et al., 2016; Lanszki et al., 2014; Carss and Parkinson, 1996; Reynolds and Aebischer, 1991; Mason and Macdonald, 1986). For example, prey remains have different retention times in the intestine, so they could appear in different spraints (Carss and Parkinson, 1996), leading to a double count of the same prey individual in different spraints. Also, it is very likely that remains of different individuals of the most consumed species are found together in the same spraint, leading to an underestimation of the importance of that species in the diet (Kruuk, 2006). Very recently, DNA barcoding has been used to identify consumed prey, but just in a

☆Fabio Marcolin and Francesca Iordan had an equal contribution in this work.
*Corresponding author
Email address: fabio.marcolin89@gmail.com (Fabio Marcolin☆)

limited number of otter spraints (N=24 in Hong et al., 2019 and N=7 in Kumari et al., 2019).

Therefore, applications of DNA barcoding to larger otter spraint samples, and particularly combined approaches using both traditional (microscope) and NGS methods to assess otter diet, could provide crucial information for the conservation of otters and the comprehension of freshwater food webs.

Otters are among the most threatened mammals in Italy (Quaglietta et al., 2019), listed in Annexes II and IV of Habitat Directive EC/92/43 and specially protected under the Italian National Law 1992/157. Recently, an otter population of unknown density has been discovered in Tarvisio municipality, in the Region of Friuli Venezia Giulia (Loy et al., 2019; Pavanello et al., 2015), several years after its local extinction, which occurred during the 1960s (Lapini, 1986). This population plausibly originated from individuals coming from the region of Carinthia (Austria), where a natural recolonization occurred in the past years (Righetti, 2011). Investigating otter trophic ecology in newly colonized areas may be particularly important to help preventing conflicts with anglers or fish farms, since contrast with these human activities have been documented (Lyach and Čech, 2017; Grant and Harrington, 2015; Klenke et al., 2013; Quaglietta, 2006, Quaglietta *pers. obs.*) and may be notably critic in areas newly colonised or recolonised by otters (Grant and Harrington, 2015; Sittenthaler et al., 2015). We used a combined approach, analysing otter spraints for the first time using both traditional (microscope) and DNA barcoding methods, to have a preliminary insight on the trophic ecology of the otter in the Tarvisio municipality. We were particularly interested in evaluating the reliability of DNA barcoding to study the diet of the otter and comparing its performance with spraints analysis through microscope. In addition, we wanted to assess whether otters in the study area prey fishes according to their availability or select certain species and/or size, to evaluate the degree of overlap, and therefore competition, with anglers. We hypothesized that otters select slower fish species and of smaller size, according to most diet studies available in the literature (Grant and Harrington, 2015; Almeida et al., 2012; Copp and Roche, 2003; Erlinge, 1968).

Materials and methods

Study area

The study was performed along the Slizza-Gailitz basin in the municipality of Tarvisio, in the Region Friuli Venezia Giulia. This northeastern Italian Region lies at the border with Austria and Slovenia (Fig. 1). The Slizza-Gailitz basin runs in a mostly mountainous landscape, with elevation ranging from 732 to 2753 m. Its 190 km^2 area encloses three glacial lakes: the Lower and Higher Fusine lakes and the Raibl lake. The area is characterized by a transaction between oceanic and continental climate (Molinari, 1998), with rigid and snowy winters and rather hot summers (Stefanuto, 2003). Mean annual rainfall is between 1400 and 2300 mm (ARPA FVG and OSMER, 2015). The landscape is characterized by a high degree of wilderness, hosting a very diverse vertebrate and invertebrate fauna (Lapini et al., 2007), except for the valleys, where anthropic pressure has profoundly modified the original landscape and depauperated the riparian vegetation (Distretto Idrografico delle Alpi Orientali, 2010). The most common fish species in the Slizza-Gailitz basin is the brown trout (*Salmo trutta*), followed by the Arctic char (*Salvelinus alpinus*) and the European bullhead (*Cottus gobio*). There are several species of amphibians, including the common toad (*Bufo bufo*), the European green toad (*Bufotes viridis*), the yellow-bellied toad (*Bombina variegata*), the European tree frogs (*Hyla arborea*), and the common frog (*Rana temporaria*). The area is the only place where the Stone crayfish (*Austropotamobius torrentium*) is known to occur in Italy (Machino et al., 2016), while there are shreds of evidence of translocation of the white-clawed crayfish (*Austropotamobius pallipes* complex) from the near catchment of the River Tagliamento (Machino et al., 2016).

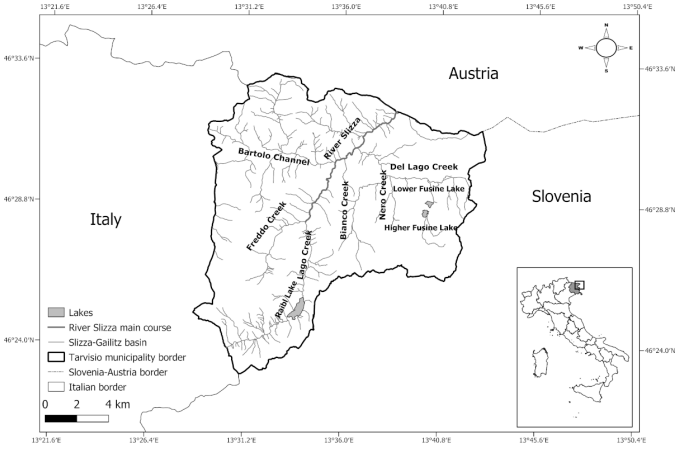

Figure 1 – Map of the study area: basin of the River Slizza-Gailitz in the municipality of Tarvisio (Friuli Venezia-Giulia, NE Italy).

Spraint collection

Spraint collection was conducted from June 2016 to February 2017. Experienced field researchers seasonally sampled the rivers of the study area following the standard sign survey method (Reuther et al., 2000), searching under "suitable bridges" (Romanowski et al., 1996). The number of selected suitable bridges was 29, including the banks of the three lakes present in the study area, and 26 bridges previously surveyed by Pavanello et al., 2015 (Fig. S1). Each spraint collected was classified as "fresh" or "old" based on appearance and colour (Mason and Macdonald, 1987), preserved in a plastic bag, and stored at −20 °C. We collected 102 spraints: 23 in spring; 28 in summer; 23 in autumn and 28 in winter.

Electrofishing

We gathered data on the composition and density of fish communities for eight permanent sampling stations in the whole Slizza-Gailitz basin belonging to the Regional Water Protection Plan network under the Water Framework Directive 2000/60/CE (WFD) scope (Fig. S1). Data of six sampling sites were provided by the Environmental Agency of the Friuli Venezia Giulia Region (Regional Water Protection Plan 2010–2015). In the remaining two sampling sites (along the Nero creek and the River Slizza), we obtained data of fish density and composition by performing electrofishing in August 2016, in a watercourse stretch whose length was proportional to the river width (Forneris et al., 2005), using the same protocol used for the Regional Water Protection Plan. The survey was performed using a battery-powered backpack electrofisher (Model IG200–2: 15–25 A, 150–200 V, manufactured by Hans-Grassl GmbH, Schönau am Königssee, Germany). Fish species were identified and the total length (TL) of individuals was measured at the nearest mm, while their total weight (TW) was measured at the nearest g. Specimens were counted, measured and released unharmed at the capture site. Density values (number of individuals per m^2) and fish biomass (g per m^2) of each station were calculated according to Ricker (1975).

Spraints analysis with the microscope

All the 102 spraints collected were analysed for prey contents using the microscope. Spraints were defrosted, soaked in water for one week (Bauer-Haáz et al., 2014; Lanszki et al., 2007) and then washed using a six-mm mesh sieve under running water, cleaning and separate the hard parts from the matrix (Conroy et al., 1993). Hard parts were then examined using a binocular microscope and assigned to the lowest taxonomy level possible, consulting identification keys for fish (Conroy et al., 1993) and amphibians (Bailon, 1999). Bones used to identify fish were: caudal vertebrae, maxillary, premaxillary, dentary, intermaxillary, palatine and preopercular. The atlas vertebrae and the praevomer bones of fishes were never found in the spraints, preventing the distinction between brown trout and Arctic char, as these are the main bones to

identify salmonids at species level (Grant and Harrington, 2015; Čech and Vejřík, 2011; Carss and Parkinson, 1996; Feltham and Marquiss, 1989). Distinction between salmonids and European bullhead was instead always possible. Not damaged fish bones were measured to the nearest mm and regression curves found in the literature were used to assess the total length of the prey (Haikova et al., 2003).

Amphibian identification was based on femur, tibia-fibula, scapula, ilion, premaxillary and maxillary bones. In general, their identification was possible only at order level, as mostly damaged bones were found. Crayfish presence was revealed by the occurrence of exoskeleton in the remains. Other remains were classified as "mammals" when hairs were found and as "other invertebrates" when elytrons, cases of caddisfly and other invertebrates remains were found. The minimum number of individuals of the same species of fish or amphibians per spraint, was calculated based on the size of the diagnostic bones and, when possible, the number of even bones found in the same spraint (Remonti et al., 2008).

Spraints analysis with DNA barcoding

Barcoding analysis was carried out on a random subsample of 50 out of 102 spraints collected. Small matrix samples were taken from the central and terminal section of each spraint, and stored at −20 °C until the analysis.

DNA extraction and sequencing

Genomic DNA was extracted with the E.Z.N.A.© Stool DNA Kit (Omega Bio-tek) according to the manufacturer's instruction, quantified with Qubit© 2.0 Fluorometer (Thermo Fisher Scientific) and stored at −20 °C until further analysis. The marker used for metabarcoding analysis is the V9 region of the 18S (e.g., Albaina et al., 2016; Jarman et al., 2013; Corse et al., 2010), a non-coding variable region of the rRNA 18S, since 1) it allows a broad diversity and richness identification (Amaral-Zettler et al., 2009), 2) it has a relative short amplicon size (160–170 bp) to be sequenced (Albaina et al., 2016), allowing the amplification also of partially degraded and shorter (<300 bp) material (i.e. DNA from faeces) (Deagle et al., 2006), and 3) it is highly redundant in organisms, having, therefore, a high probability of being amplified (Albaina et al., 2016; Corse et al., 2010).

Amplifications were conducted with the primer system 1391F and 1795R EukB (Edgcomb et al., 2011) tailed with Tail_1 and Tail_2, respectively (Carew et al., 2013). The thermal profile for the PCR amplification included an initial denaturation at 95 °C for 1', followed by 25 cycles at 95 °C for 30", 58 °C for 45" and 72 °C for 45 seconds, and a final extension at 72 °C for 5'. The PCR products were checked lately in TBE agarose gel 2.5%. A secondary PCR was performed for barcoding samples and a pool of equimolar products was emulsion-amplified with Ion PGM Hi-Q^{TM} OT2 400 kit (Thermo Fisher Scientific) and then sequenced with Ion PGM Hi-Q^{TM} sequencing kit on a 316 V2chip in an Ion Torrent PGM.

Statistical analysis

Microscope data

Each prey taxon identified was considered an occurrence. Results of the diet analysis were expressed as relative frequency of occurrence (RFO%): (number of occurrences of a given prey taxon / total occurrence of all prey taxa in the 102 spraints) × 100 (Conroy et al., 1993). Two values of RFO% were calculated: one account for all the fish, amphibians and crayfish taxa identified, for a total of three taxa, and the second where fish were separated in the two most represented taxa, for a total of four taxa (i.e. salmonids, European bullhead, amphibians, and crayfish). RFO% values were calculated on annual and seasonal basis.

We assessed difference in the diet among seasons using the χ^2 of independence test and variation in the TL of salmonids preyed between seasons using the Kruskal-Wallis test. The limited data collected for European bullhead did not allow robust statistical inference on the prey-predator interaction with otters. Thus, we focused our attention mainly on Salmonids, since they are present at high densities in the Slizza-Gailitz basin (Stoch et al., 1992) and are the only fish species of angling interest in this area. Mammals and invertebrates (excluding crayfish) were counted only as presence or absence because their occurrence was low compared to the other preys, and because they occur only occasionally in the diet of Eurasian otters in Europe (Clavero et al., 2003).

Normal distribution and homogeneity of variance of the data were tested using Shapiro-Wilk and Bartlett tests, respectively.

Salmonids size class selection

To assess whether otters selected specific size classes of salmonids we compared the data obtained from the electrofishing, which represented the "availability", and those from the spraint analysis, representing the "use" (Manly et al., 2007), using the χ^2 test. We further assessed otter prey selection using the Standardised Selection Index (B_i) (Grant and Harrington, 2015; Krebs, 1999), to examine which size classes were consumed more than expected. This index is based on the Selection Index described by Krebs (1999), which is often used in dietary study (Grant and Harrington, 2015), and it is calculated as: $w_i = \frac{o_i}{p_i}$, where w_i is the Selection index of resource i, o_i is the proportion of resource i in the diet, and p_i is the proportion of resource i in the environment. The Standardised Selection Index is then calculated as: $B_i = \frac{w_i}{\sum_{j=1}^{n} w_j}$, where B_i is the standardised selection index for resource i, w_i is the Selection index of resource i in the diet, and w_j is the Selection index of each resource j in the diet.

An index of "availability of resources" (A) was calculated as 1/number of resources, and turned out to be equal to 0.11 because the "number of resources" in this study is nine (i.e. specific size classes of salmonids). The comparison of B_i with A allows to highlight the selection or the avoidance of certain prey classes by otters: values of B_i approximately equal to A indicate that there is no relative selection; B_i values higher than A indicate relative selection; B_i values lower than A indicate relative avoidance (Grant and Harrington, 2015).

Barcoding data

For the processing of the raw data, the software MALT v.0.4.1, in semi-global alignment has been used (Herbig et al., 2016) with the default parameters except for the length of the reads (reads below 95 bp were discarded). Sequences that passed the quality checks have been aligned and metagenomically analysed using a GenBank database as a reference (data obtained from www.ncbi.nlm.nih.gov) built with sequences from Vertebrates (taxid: 7742) and Decapoda (taxid: 6683), clustered with CD-HIT at 99% (Fu et al., 2012).

Whenever possible, we identified the taxon at species level, considering the identity of each read at two different levels: 100% and 99% of similarity between the sequence of a certain taxon found using the barcoding analysis and the sequence of the same taxon present in the reference database. For each taxon, average and standard deviation of the reads per each spraint were calculated. The correlation of the number of reads per spraint between different taxa was tested with the Spearman's correlation index. All statistical analysis was carried out using the software R statistics (version 3.0.2, code Frisbee Sailing).

Results

Spraints collection

Out of the 102 spraints collected, 90 were classified as "old" (22 in spring, 24 in summer, 16 in autumn and 28 in winter), and 12 as "fresh" (one in spring, four in summer and seven in winter). The number of bridges under which at least one spraint was found was 11 (38% of the "suitable" and 19% of the total bridges, respectively) (Fig. S1). Only two spraints were found along the banks of the Lower and Higher Fusine lakes.

Electrofishing

Electrofishing provided a total of 315 fish individuals. Salmonids represented the majority of the fish detected, of which 82.2% were brown trout, while the Arctic char (8.9%) and the European bullhead (8.9%) accounted equally for the remaining 17.8% (Tab. S2). The Arctic char

Table 1 – Minimum number of individuals of different taxa found in spraints and considered in the diet analysis.

	Season				
Taxon	**Spring**	**Summer**	**Autumn**	**Winter**	**Total**
Salmonids	37	32	32	45	146
E. bullhead	3	15	12	11	41
Amphibians	15	10	6	6	37
Crayfish	2	2	2	0	6

was captured only along the stream Lake creek, and the European bullhead only at the northern station of the River Slizza (Fig. S1). Average TL of the captured salmonids was 178±60 mm, while the average TL of the captured European bullhead was 112±15 mm. Average fish biomass per sampling station was $2.09 \pm 2.20\,g\,m^{-2}$ varying from $0.11\,g\,m^{-2}$ (River Slizza) to $5.93\,g\,m^{-2}$ (Bianco creek).

Diet analysis

Spraint analysis with the microscope

Fish was the main prey category occurring in the spraints (RFO% = 70%), followed by amphibians (RFO% = 26%) and crayfish (RFO% = 4%) (N=102) (Fig. 2). Among the two fish groups identified, salmonids were more consumed than European bullheads (*Cottus gobio*) (Fig. 3). Amphibians were identified at species level in 10 cases (out of 37 total amphibian occurrences): nine common frogs and one common toad (respectively 24% and 3% of the total amphibians remains). Crayfish were assigned to the genus *Austropotamobius*, which is the only one known to occur in the area (Machino et al., 2016). We could not distinguish with accuracy between *Austropotamobius pallipes* complex and *Austropotamobius torrentium*, since the rostrum, which allows the discrimination between the species (Mazzoni et al., 2004), was never found in the spraints. The total minimum number of prey individuals found in spraints was 230, with an average of 2.25 ± 1.08 prey individuals per spraint, and a maximum of 6 prey individuals in one spraint (Tab. 1).

Salmonids (and fish as higher taxon) turned out to be the main component of the diet of otter in each season (Fig. 3a,b).

European bullheads showed a seasonal variation, increasing from spring to autumn (with a slight decline in winter) (Fig. 3b), while amphibians showed an opposite trend (Fig. 3). In each of the considered seasons, amphibians were more represented in the diet of otter than the European bullhead (Fig. 3). Diet composition did not vary among seasons, considering either three (fish, amphibians, crayfish, χ^2=12.10, df=9, p=0.21) or four (salmonids, European bullhead, amphibians, crayfish, χ^2=8.91, df=6, p=0.18) prey taxa.

Prey size analysis

Salmonids were the largest fish consumed, with an average TL of 135 ± 50 mm (N=146), while European bullhead had an average TL of 76 ± 20 mm (N=41). The size class of salmonids consumed did not vary among seasons (Kruskal-Wallis test: χ^2=2.79, df=3, p=0.42, N=146).

Standardized Selection Index of salmonids size classes

Salmonids size classes found in spraints differed from those revealed through electrofishing (χ^2=1012.659, df=8, p<0.01) (Fig. 4). According to the B_i (Standardized Selection Index), otters positively selected the size class of 100 mm (B_i=0.78), consumed the size class 125 mm according to its availability (i.e., without relative selection — B_i=0.11), and relatively avoided all other class sizes (B_i<0.11) (Tab. 2).

Spraints analysis with DNA barcoding

Extraction and amplification of DNA succeeded in all the spraints analysed (N=50), regardless of the type of spraint ("fresh" or "old"). The reference database included 249409 sequences of the 18S-V9 from both vertebrates and decapods (similarity percentage of 99%). Following the quality check of the reads using MALT alignment the most significant number of reads belonged to the family of salmonids (99% of total reads, 173083 out of 173607, 100% and 99% identity; Tab. S3). Among salmonids, barcoding identified the Salmoninae subfamily (96%, 165978 out of 173083 reads, identity between 100–99%) and the species brown trout (4%, 7105 reads out of 173083 reads, identity between 100–99%). Amphibians were identified at superorder level (Batrachia, 438 reads in 15 spraints, identity between 100–99%). Some reads were assigned to the otter itself (total of 1679 reads at 99% identity) and to taxon that do not occur in the study area, like the family Emydidae (total of 86 reads at 99% identity). A total of 75802 reads (at 99% of identity) were not found in the reference database and thus were not assigned. The number of reads per spraint of the taxa Salmoninae and of the brown trout were highly correlated (ρ=0.99, p< 0.0,1, S=138.01) (Tab. 3).

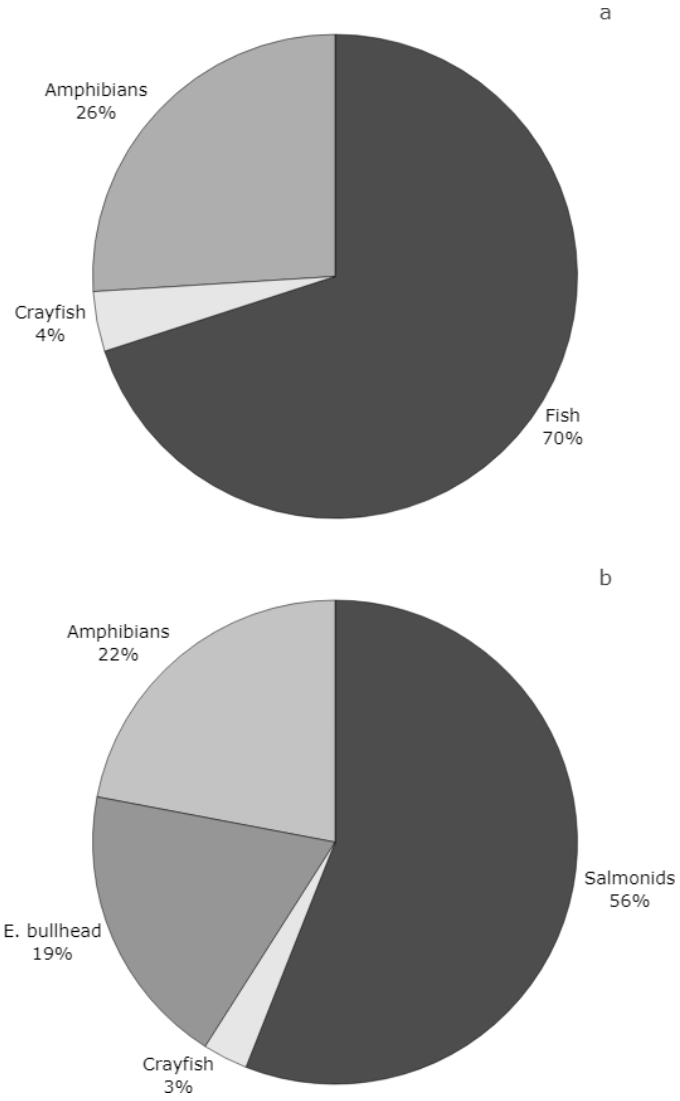

Figure 2 – Annual Relative Frequence of Occurrence (RFO%) of preys found in spraints (N = 102) using the microscope. a: annual RFO% of preys aggregating taxa (salmonids and E. bullhead together). b: annual RFO% of prey with the fish taxon splitted into salmonids and E. bullhead.

Comparison of diet analysis methods

Fifty spraints were analysed using both the barcoding and the microscope method. Barcoding failed to detect the European bullhead and the crayfish, while these two preys were detected using the microscope (European bullhead being identified at species level in 16 spraints and crayfish at genus level, *Austropotamobius*, in two spraints). On the other hand, the family Emydidae was detected only by the barcoding method in 24 spraints.

Salmonids identification at species level was possible only through barcoding (i.e. brown trout = 98% of spraints analysed, N=50), while

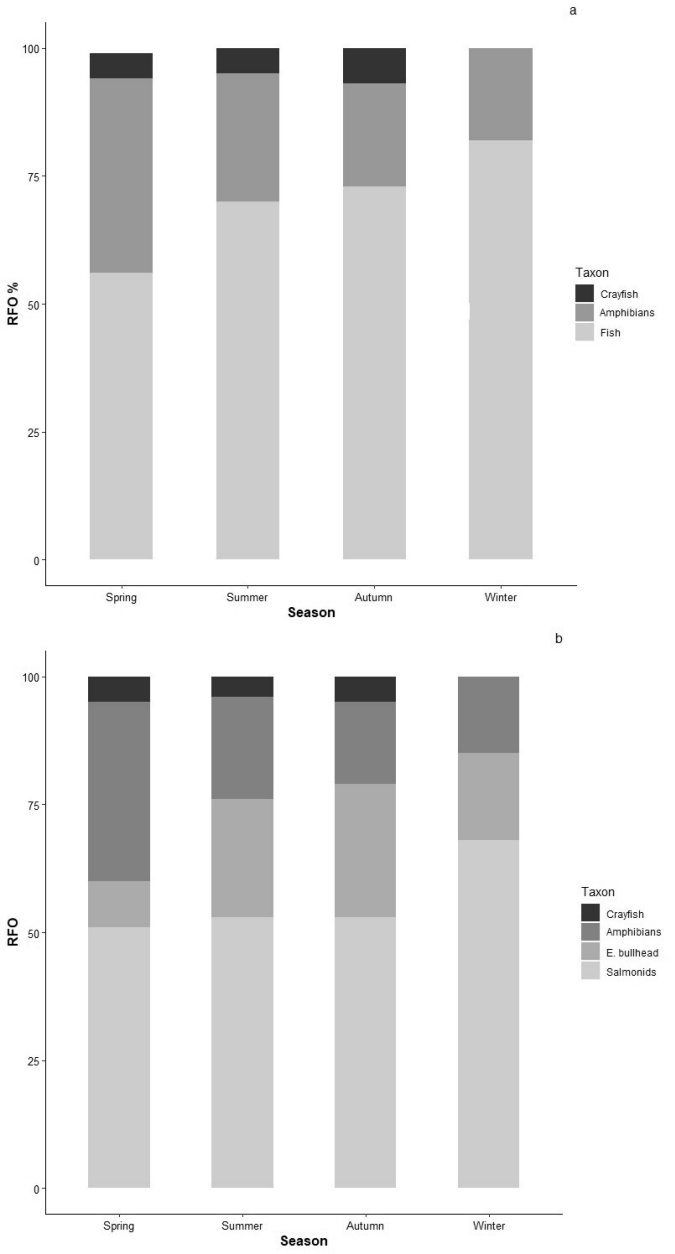

Figure 3 – Seasonal Relative Frequence of Occurrence (RFO%) of preys found in spraints (N = 102) using the microscope. a: Seasonal RFO% of preys with aggregated taxa (salmonids and E. bullhead together). b: seasonal RFO% of preys splitting the fish taxon into salmonids and E. bullhead.

the identification at family level was obtained with both methods (salmonids: barcoding method = 100%, N=50; microscope method = 98%, N=50). Amphibians were identified both at species (10%, N=21) and order level (amphibians = 90%, N=21) only with the microscope analysis, whereas the barcoding allowed identification only at superorder level (Batrachia = 100%, N=15). By considering only salmonids, the correspondence between the two methods was 98% (i.e. in 49 out of the 50 spraints the two methods provided the same results) (Fig. 5a,b and Tab. S4), while considering salmonids and amphibians the correspondence between the two methods fell to 68% (i.e. in 34 out of the 50 spraints results obtained with the two methods were the same.). When considering all the taxa (salmonids, amphibians, European bullhead, crayfish, Emydidae) (i.e. 15 out of 50) the two methods showed a correspondence of 30%.

Table 2 – Standardised selection index (B_i) of size classes of salmonids consumed by otters. Size class represents the nine possible salmonids classes considered in this study. Sample proportion is the proportion of each size class detected in otter spraints (N = 102). Standardised selection index (B_i – see main text) > (0.11 in this case) indicates relative selection for that particular size class; B_i = 0.11 indicates no relative selection for that particular size class; B_i < 0.11 indicates relative avoidance for that particular size class. o_i is the proportion of resource i in the diet, p_i is the proportion of resource i in the environment.

	Environment proportion (p_i)	Sample proportion (o_i)	Standardised selection index (B_i)
51-75	0.15	0.08	*0.01
76-100	0.01	0.23	*0.78
101-125	0.03	0.16	0.11
126-150	0.22	0.19	*0.02
151-175	0.13	0.12	*0.02
176-200	0.11	0.10	*0.02
201-225	0.15	0.06	*0.01
226-250	0.16	0.03	*0.00
251-275	0.04	0.03	*0.02

* size classes with relative selection or avoidance.

Table 3 – Average number of reads per spraint (N = 50) +- standard deviation for the main prey taxa found trough DNA-barcoding analysis. Values are reported for two identity thresholds.

		Taxon		
	N°reads Salmoninae	*Salmo trutta*	Batrachia	Emydidae
100% identity	2979 ± 2751	142 ± 132	7 ± 20	0
99% identity	341 ± 313	0	1 ± 4	2 ± 3

Discussion

Microscope analysis

The main fish prey for otters in the area of Tarvisio resulted being salmonids, while the European bullhead appeared less frequently in the otter diet. This is in contrast with other studies reporting the European bullhead as a more frequent prey than salmonids in those areas where the two taxa are sympatric (Grant and Harrington, 2015; Brzeziński et al., 2006; Thom, 1990). According to the optimal foraging theory (MacArthur and Pianka, 1966), predators should select prey species that provide the highest energetic input per time unit. Fast-swimming and large species as salmonids are more challenging to catch and handle than small and slow-swimming species as European bullheads. Therefore, where European bullheads occur at high density, they usually are the most fitting trophic resource for otters (Grant and Harrington, 2015; Taastrøm and Jacobsen, 1999). At the same time otters

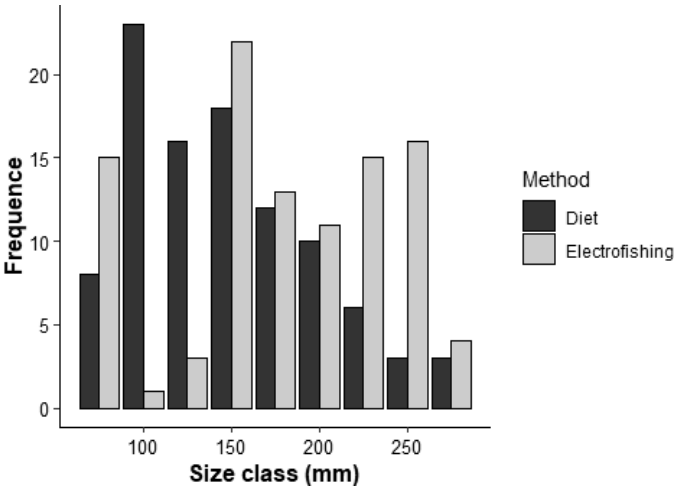

Figure 4 – Comparison of the size class and relative frequence (%) of salmonids found in spraints (dark grey) and sampled by electrofishing (light grey).

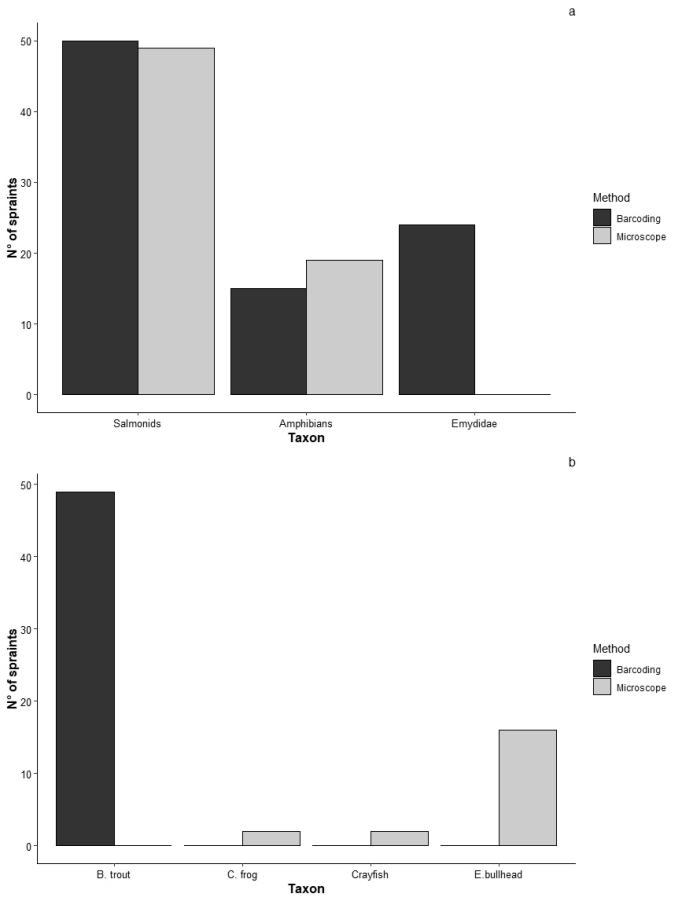

Figure 5 – Comparison between the 50 spraints analysed with both microscope and DNA barcoding. a: prey identification at family or superior level. b: prey identification at species or genus level. B. trout = brown trout, E. bullhead = European bullhead, C. frog = common frog.

also tend to prey mainly on the most abundant and available species (Remonti et al., 2009; Clavero et al., 2003; Taastrøm and Jacobsen, 1999; Chanin, 1981), behaving as opportunist predators with great foraging plasticity (Almeida et al., 2012). Brown trout are abundant in our study area, due to restocking programs for angling purposes (data provided by the Fish Resource Safeguard Authority of Friuli Venezia Giulia), whereas the European bullhead is an introduced species that in the area occurs at low density and only in some watercourses (Stoch et al., 1992). Thus, otters in our study consumed more salmonids than European bullheads probably due to the overall higher density of the formers. Moreover, several trout in the area stem from hatchery programs, and therefore have poor swimming skills (Sittenthaler et al., 2015) and predator avoidance behaviour (Petersson and Järvi, 2006; Olla et al., 1998), constituting an easy prey for otters.

Diet composition was stable among seasons, as documented by others (Karamanlidis et al., 2014; Brzeziński et al., 2006; Chanin, 1981). Otter diet is generally homogeneous and more stable in oligotrophic streams and lakes characterized by only a few fish species and with salmonids being the most abundant resource available compared with other aquatic habitats with a more diverse ichthyofauna (Brzeziński et al., 2006; Chanin, 1981). The above conditions are met in our study area, where native and restocked salmonids represent an abundant resource throughout the year.

The majority of the fish consumed (salmonids and European bullheads) were of small-medium size, in accordance with other European studies (Grant and Harrington, 2015; Kruuk, 2006; Lanszki et al., 2001; Taastrøm and Jacobsen, 1999). Most of the salmonids individuals consumed was between 76 and 175 mm. Similar results were obtained by Lyach and Čech (2017) and by Miranda et al. (2006), who reported otters eating mainly salmonids between 80 and 160 mm and 90 and 180 mm, respectively. The size classes of salmonids detected in the spraints differed from those observed in the environment through electrofishing. In particular, the Standardized Selection Index suggested a relative selection for the salmonids of size class 100 mm, during all the year.

Otter high consumption of small- to medium- sized fish is thought to be due to the generally high availability of these size classes (Clavero et al., 2007; Lanszki and Sallai, 2006; Britton and Shepherd, 2005), and their higher catchability, compared to bigger fish, which would make the former energetically more convenient than the latter (Miranda et al., 2006; Ruiz-Olmo et al., 2001). Furthermore, high otter consumption of juvenile salmonids could also reflect the habitat preference of this fish taxon. In fact, juvenile salmonids generally inhabit different sectors of rivers compared to adult salmonids, preferring gravel-sized substrate and shallower water (Johnson, 2008; Bley, 1987), which have shown to be among otters preferred places to hunt (Quaglietta, 2011; Kruuk, 2006; Carss et al., 1990; Erlinge, 1969).

Otters appeared to avoid size classes of salmonids between 225 and 275 mm. Nevertheless, we cannot exclude that they did not ingest vertebrae or other bones of big-sized fish, leading to an underestimation of these size classes in the spraints (Lanszki et al., 2007; Adámek et al., 2003; Carss et al., 1998; Carss and Parkinson, 1996).

Overall, otters fed mainly on salmonids of smaller size than the minimum size catchable for anglers (i.e. 220 mm for brown trout and 300 mm for Arctic char). There is, therefore, no or minimal overlap between the fish consumed by the otters and those of angling interest in the study area, which should help to minimize the risks of conflicts between otters and humans. To better understand the impact of otters on angling activity, however, we advocate for further data from electrofishing surveys and otter diet before and after the release of stocked salmonids (Jacobsen, 2005).

Limited fish abundance or its seasonal variation (when streams becomes dry, particularly in Mediterranean areas) may lead significant presence of crayfish and amphibians in the diet of otters (Quaglietta et al., 2018; Krawczyk et al., 2016; Remonti et al., 2010; Clavero et al., 2003). In our study, amphibians were consumed much less than fish, and crayfish even less. Otters may prefer fish to amphibians, because these have lesser energetic content than fish (Nelson and Kruuk, 1997), as well as due to the time that they have to spend to avoid toxic parts of certain amphibians (Grant and Harrington, 2015). Similarly, crayfish are thought to be less preferred by otters because their protective integument needs to be broken before otters can eat the edible part, thus providing a low energetic return on the energy expended in hunting (Grant and Harrington, 2015).

The consumption of amphibians decreased from spring to winter (Fig. 3). The spring peak of catches may be related to the greater vulnerability of amphibians during the spawning period (Clavero et al., 2005; Lanszki et al., 2001; Sidorovich and Pikulik, 1997; Weber, 1990) when they aggregate in large numbers (Duellman and Trueb, 1986). On the other hand, the lower consumption in winter may be related to otter higher predation of fish during this season. This has shown to be the consequence of the lower water temperature in this season, which slows the fish metabolism reducing swimming speed (Wardle, 1980), thus enhancing fish vulnerability to otter predation (Grant and Harrington, 2015). We did not detect crayfish remains in the spraints collected in winter, possibly because crayfish reduce their activity in winter, burrowed in river banks (Bubb et al., 2002), thus lowering the probability to be preyed by otters (Grant and Harrington, 2015).

DNA barcoding analysis

We successfully amplified a short DNA sequence (157 bp) in all the analysed samples (both "fresh" and "old" spraints). The results provided by the marker we chose (18S-V9), however, were somewhat limited, due to the lack of reference sequences in the GenBank database for many potential prey species occurring in the study area and for the low resolution of this molecular barcode. For all spraints, a total of 75802 reads (at 99% of identity) were not assigned while others could have been wrongly assigned (family, genus or species) based on the similar-

ity between the sequence obtained in our study and those present in the online database.

Most of the assigned reads belonged to the family of salmonids, with a few specific assignments to the brown trout and the majority to the subfamily Salmoninae (Tab. S3). The number of reads assigned to the Salmoninae and the brown trout were highly correlated, suggesting that all the Salmoninae reads could belong to the brown trout. However, we cannot assume this, since the Arctic char, also belonging to the Salmoninae subfamily, occurs in the study area and may have gone undetected because there were no sequences of the marker 18S-V9 on the online database for this species. Moreover, some authors state that the 18S-V9 is characterized by low taxonomic resolution (Zhan et al., 2014; Tang et al., 2012), and therefore could not allow distinguishing with certainties two phylogenetically closely related species, such as the brown trout and the Arctic char. However, according to Albaina et al. (2016), the marker 18S-V9 performs well and has proper taxonomic resolution, as long as the sequences needed are present in the reference database. Most likely, such lack also explains why the DNA barcoding analysis missed the detection of the European bullhead. Therefore, the use of a reference database, including all the potential local prey species should be implemented, since it represents a key aspect for a reliable DNA barcoding analysis (Albaina et al., 2016).

We observed 86 reads assigned to the family Emydidae. This taxon in Friuli-Venezia Giulia is represented by the native European pond turtle (*Emys orbicularis*) and the invasive pond slider (*Trachemys scripta*) (Lapini et al., 2007). Both species have not been yet reported in the study area, even though illegal releases of pond slider cannot be excluded (Lapini et al., 2007). Otter predation upon European pond turtles has been reported in the literature (Lanszki et al., 2009; Lanszki and Lanszkiné Széles, 2006), although otter consumption of this prey is generally low and mostly associated to particularly cold winters (Lanszki and Lanszkiné Széles, 2006). We did not detect turtle by microscope. Thus, otter predation on turtles in the study area is inconclusive for the moment.

No crayfish were found by the DNA barcoding analysis. As discussed above, the failed detection of this taxon can be due to the lack of the crayfish sequence in the online database of the marker used.

All the mammals reads found (1679 at the 99% of identity, 0.67% of all the reads) were assigned to a portion of the 18S rRNA of *Lutra lutra* isolated in this study from the recently published otter genome assembly, matching from 47355493 to 47355826 bp (LR738418, Bio project PRJEB35339). In this genomic assembly the whole ribosomal cluster (from 18S to 28S) goes from 47346205 to 47355847 bp. The high abundance of otter's reads is due to the release of epithelial cells and hairs (grooming behaviour; Kruuk, 2006) in their spraints.

Limitations of the study

The most critical issue in our study lies in the lack of local and updated reference database including sequences of potential otter preys. While the DNA barcoding was able to attribute some salmonids sequences to the brown trout (*Salmo trutta*) allowing a more accurate identification than the microscope, as reported in other studies (Dahl et al., 2017; Lu et al., 2016; Santos et al., 2015; Jarman et al., 2013; Shehzad et al., 2012; Zeale et al., 2011), the microscope analysis showed better accuracy regarding the identification of European bullheads, amphibians (e.g. common frog) and crayfish. Other studies on the diet of carnivores using DNA barcoding analysis documented better accuracy of this method over the microscope analysis, but such studies used different markers than us: 12S rRNA in Santos et al. (2015) and Shehzad et al. (2012), 16S rRNA in Deagle et al. (2009). Nonetheless, Albaina et al., 2016 had considerable prey species identification accuracy using the marker 18S-V9. Another limitation lays on the impossibility, of both methods, to distinguish between individuals, belonging to the same species, found in the spraints, thus leaving unresolved the issue of under/overestimating some species in otter diet (Kruuk, 2006; Carss and Parkinson, 1996).

Conclusions

Based on our results, to accurately identify all the potential prey consumed by otters (i.e., including amphibians, crayfish, mammals, etc.), sequencing other markers (COI, 12S, 16S – see Smith et al., 2018; Lu et al., 2016; Mata et al., 2016; De Barba et al., 2014; Shehzad et al., 2012; Deagle et al., 2009), or combining set of markers (see Kumari et al., 2019), would be recommendable. Therefore, signals that in this study could have been masked by low sequencing depth could be unveiled by deeper sequencing in future work.

Recently, Hong et al. (2019) and Kumari et al. (2019) applied for the first time DNA barcoding (marker 12S and 12S, 16S, COI respectively) to study otter diet. Their conclusions, however, were hampered by the limited sample size (N = 24 – Hong et al., 2019; N = 7 – Kumari et al., 2019). Also, in Hong et al. (2019) the authors amplified the DNA only from bones, missing the DNA information enclosed in the spraints matrix.

Although this study had an exploratory character of the methodologies, we provided the first hint on the positive application of both microscope and DNA barcoding analysis to study otter diet composition. Nonetheless, this synergic approach seems promising, minimizing the limitations of the individual methods (Lu et al., 2016), with the DNA barcoding significantly improving the "qualitative" information on species identification, and the microscope analysis allowing information on prey size (and, potentially, biomass). Further assessing such combined approach would be beneficial to deepen the knowledge on vertebrates feeding ecology.

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Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Figure S1 Distribution of suitable bridges and electrofishing sampling sites in the study area.

Table S2 Number of fish (N=315) individuals provided by electrofishing.

Table S3 Number of reads assigned (100 and 99% of identity) per each spraint (N = 50) and each taxon.

Table S4 Comparison of results with both methods.