MICROPLASTICS, ALIEN SPECIES AND AMPHIBIAN MALE-SPECIFIC PREDATION IN RIVER OTTER DIET (*Lutra lutra*). A STUDY OF TWO POPULATIONS IN THE TICINO VALLEY (NORTH ITALY) AND SILA MASSIF (SOUTH ITALY).

Giorgio Smiroldo PhD Thesis

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Ph.D. Thesis
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Anno accademico 2016-2018

I dedicate my PhD to my mother. And to my father notwithstanding not here anymore.

Giorgio Smiroldo - Pencil on paper 33x24 cm (2010)
Private collection

Abstract

Distribution and diet of Eurasian otter (*Lutra lutra*) was studied in two different Italian areas: in the North (Ticino River) and in the South (streams from Sila Massif). The Ticino population derives from a reintroduction project performed by the two Park Autorities in the 90', while the Sila Massif population are probably the descendent of relictus populations. The Ticino population is characterised by few founders coming from European reproduction centers. The release, in 1997, of a pair of otters *Lutra lutra*, from the breeding enclosure centre of Cameri "Bosco Vedro" (Novara) and after the escape during a flood of two adult and a cub in 1993 from the breeding centre "La Fagiana" (Magenta), create a little vital otter nucleous in the valley. The survey performed in 2010 provideed a small range of otter activity (7 km near the breeding centre of Cameri), in 2012 an otter sighting has been recorded and in 2013 an individual has been found dead in Southern stretch of the Ticino River near Pavia, about 30 km downstream the release site. Seven years after the last monitoring, a new survey was performed along all the Ticino River. Status and distribution of the Ticino's otters showed a surprising evolution of its distribution. 101 feces samples were recorded in 16 stations on the 32 survayed (50%). Positive stations covered near the whole watercourse from Lake Maggiore to Pavia for a length of 100 km of river. In otter diet, the allochtonous european catfish (*S. glanis*) has been recorded in Italy for the first time. The high incidence of this species in the otter diet reflects its demographic explosion in Ticino River and suggests river otter such potential natural predator to control the exponential diffusion of this alien fish. To quantify the presence of *S. glanis* in the otter diet, morphometric equations were developed basing on 28 *S. glanis* speciemens, coming from the same area. Regression equations relate the known fish mass to skeletrical element dimension. Two diagnostic elements were selected starting from those found in otter stools: vertebrae and a basicranial bone. A second unexpected result found in otter feces from Ticino River was that, unfortunality, for the first time, spectroscopy analysis performed on some unknown remains in otter stool showed theirs correlation with microplastics. Probably the mustelid ingested these particles not by directly ingestion but through fish preyed. Among the multiple human pressures on aquatic ecosystems, the accumulation of plastic debris is an increasing environmental problem in marine and freshwater system. The transfer of microplastic pollution across trophic levels has been proven in marine ecosystems such between mussels and crabs. The similarity between ecological niches and feeding strategies for species in marine and freshwater environments would suggest that trophic transfer should also occur in freshwater ecosystems.

Considering toxicological problems related to additives release and adsorbed pollutants, this result rises a new treat for otter conservation, and certainly requires further studies.
South Italy represent for Italian otter population the main core. Although in 20^{th} century the mustelid suffered an important constriction of its range also here, recent works showed an expansion in this zone as elsewhere. The south Mediterranean area is a delicate ecological environment characterized by extreme seasonal variations in water flow with dry and harsh period in summer and torrential floods usually occuring in autumn and spring. This conditions could make the trophic resources and the river accessibility for otters highly fluctuating. Strategies for facing fluctuations in habitat accessibility and food resources are essential for the lifestyle of otter which is metabolically costly and strictly linked to aquatic habitats. We analysed the southern Italian range on 8 streams flowing from the Sila massif, and we found a stable and positive trend of the otter presence. 357 otter stools were found from 2014 utill 2017 principally in Savuto, Amato, Lese and Neto rivers. Diet analysis confirmed an increase of trophic diversity from northern to southern Europe with a high proportion of amphibian consumption. Usually diet analyses focuses on fish preys, while amphibians are lees detailed with only one or few categories. Little number of studies classified amphibians to the species level. The high presence of amphibians in the present study induced us to perform an accurate species and genus analysis. Osteological identification of amphibian remains allowed to ascertain otter predation on seven out of the 11 species recorded for the study area. Particularly interesting was the discovery of newts in otters' feces for the presence of the powerful neurotoxin in skin secretion of these urodelans. Furthermore, osteological analysis provided a male-biased predation by otter on Anurans.
Stool analysis provided further interesting insights on the feeding behavior of otter. By the analysis of unknown remains performed with Attenuated Total Reflectance (ATR-FT-IR) spectroscopy, the 61% of these materials showed peaks typical of protein and high correlations with amphibian eggs.
According to other studies, results of this thesis showed that Italian otter populations are following a positive trend of expansion both in the North than in the South. The diet results highlighted the plastic feeding behavior of otter, through which otters feed on the most accessible prey: alien species in the North and amphibians, even newts, in the South, with a male specific predation. The presence of amphibian eggs in otter stools may derive from the ingestion of females before spawning.

Contents

1. Introduction

1.1 Distribution and status of the river otter (Lutra lutra).

Lutra lutra is the most widely distributed otter species among the 13 existing in the world, with 12 subspecies occurring throughout Asia, all of Europe, and parts of northern Africa (Fig. 1). Historical range originally extended from Japan in the East to Portugal in the West, and from the Arctic regions of Asia and Europe to as far as Indonesia in the South (Foster-Turley et al. 1990). Its current distribution in Europe is marked by a large corridor, stretching from central Denmark, via the western parts of Germany, the Netherlands, Belgium, Luxembourg, the eastern parts of France, Switzerland, the western parts of Austria to central Italy, where the otter is extinct or reduced to small and sometimes isolated subpopulations (Roos et al. 2015).

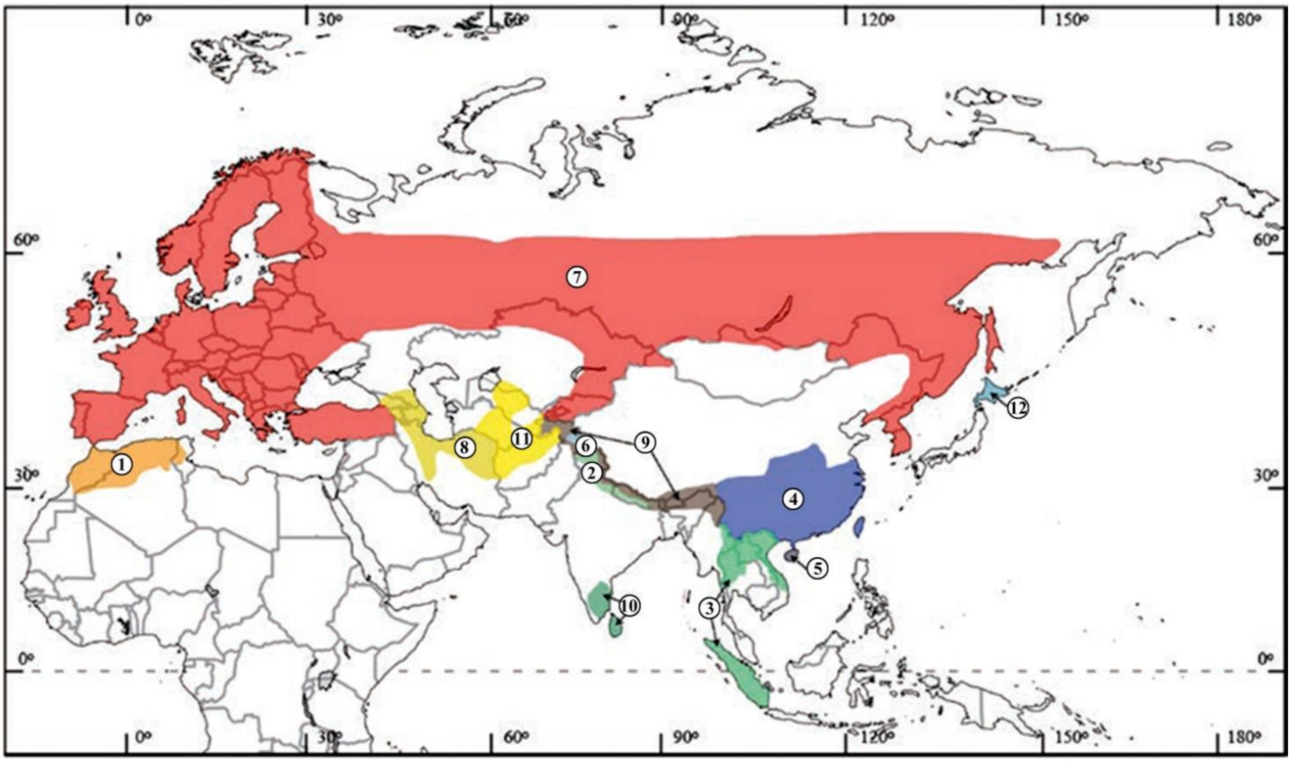

Fig. 1. Geographic distribution of *Lutra lutra*. Map redrawn from (Romanowski et al. 2010). Subspecies: 1 *L. l. angustifrons*; 2 *L. l. aurobrunnea*; 3 *L. l. barang*; 4 *L. l. chinensis*; 5 *L. l. hainana*; 6 *L. l. kutab*; 7 *L. l. lutra*; 8 *L. l. meridionalis*; 9 *L. l. monticolus*; 10 *L. l. nair*; 11 *L. l. seistanica*; and 12 *L. l. whiteleyi*.

During the 20th century, this mustelid in Europe declined dramatically, especially in Central and Western European countries (Ruiz-Olmo et al. 2008). This strong decrease was mainly due to water pollution, with intensification of surrounding agriculture resulting in the alteration of water chemistry (Gutleb and Kranz, 1998; Kruuk, 1995), poaching, increased road traffic, and habitat loss. Although extant populations remain widespread, *L. lutra* numbers, particularly in Europe, continue to be threatened due to environmental pollution, habitat fragmentation, accidental trappings in fishing nets (Koelewijn et al. 2010) or in traps designed to kill other species (e.g. cages constructed to drown muskrats), illegal and legal hunting, indeed in several European countries fishermen political pressure who brought in particular to the licenses for killing otters (Reuther and Hilton-Taylor 2004). Besides, river modification (Panzacchi et al. 2010; Scorpio et al. 2014) growing water demands, threatened freshwater species, with extinction rates that are four to six times higher than their terrestrial counterparts (Magurran, 2009). Due to this significant decline throughout Europe since the 1950s this mustelid is classified as 'near threatened' by the IUCN Red List with a decreasing population trend and, as such, is listed in Appendix 1 of CITES, Appendix II of the Bern Convention (Council of Europe, 1979) and Annexes II and IV of the 'EC Habitats & Species Directive' (92/43/EEC). According to the precautionary principle, since the population it is certainly less than 1000 individuals, and the number of reproductive individuals it is less than 250 (Prigioni et al. 2006a; 2006b), the otter is listed in the Italian Red List of endangered species as "Endangered" (EN), according to IUCN criteria (IUCN 2001).

Recently, river otter has been reintroduced to areas where its population remains in decline, such as the Netherlands (Koelewijn et al. 2010), Spain (Saavedra and Sargatal 1998), Sweden (Sjöåsen 1996), Switzerland (Conroy and Chanin 2000), and in specific areas in Britain (Mason and Macdonald 2004). In the latter, population annual growth rate was of 1–7% (Mason and Macdonald 2004 In Italy, the otter became extinct in northern part and further declined in the central part of the peninsula. Recently signs of a recovery were recorded in southern Italy (Fig2), with a positive trend for four regions: Campania, Basilicata, Apulia and Calabria (Prigioni et al. 2007). Furthermore, recently otter activityhas been recorded in the North-Eastern of the peninsula, as a consequence of the expansion of Austrian and Slovenian populations; otter signs have been found since 2008 in South Tyrol (Kranz 2008), and nowadays the species occurs on the Italian stretch "Rio Sesto" (Righetti 2011). Otter spraints were found also on the Natisone River, Friuli Venezia Giulia region (Lapini and Bonesi 2011). According to the recent expansion of the species on 15th August 2012 in Lombardy region (North Italy) a 4 year-old male otter was found road-killed in loc. Tovo di Sant'Agata (Sondrio), in the overlying Catchment of the River Adda.

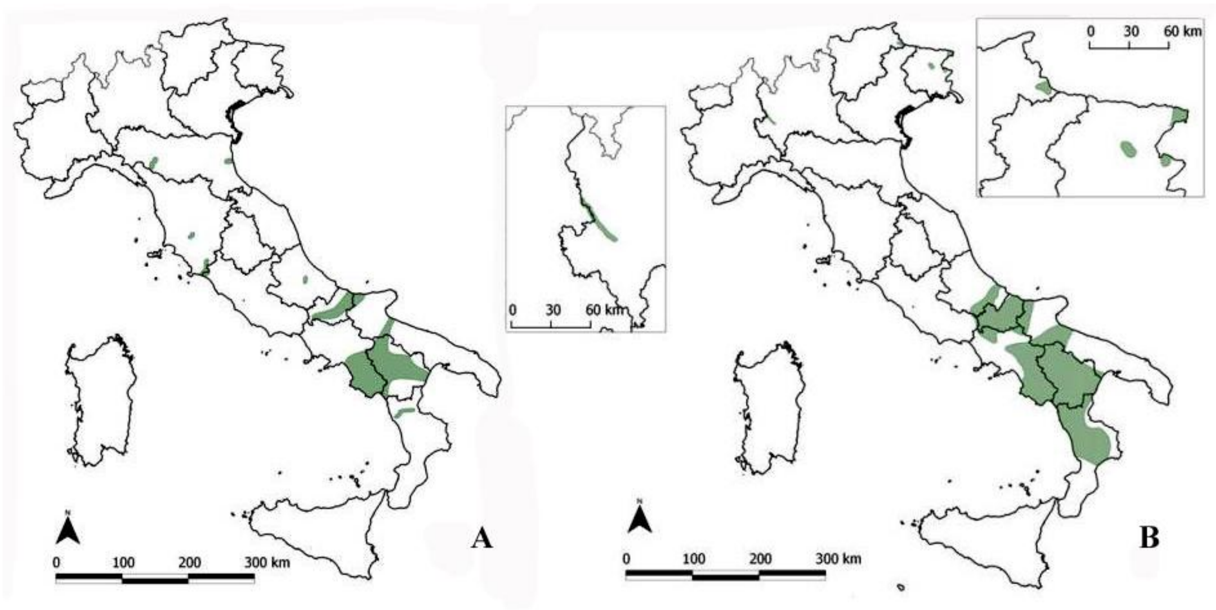

Fig2 A: Otter range in the Italian peninsula in the 1980s (left); **B:** Current otter range in the Italian peninsula with enlargements of the River Ticino Valley (left) and South Tyrol (Trentino Alto Adige region) and Friuli Venezia Giulia region (right). Modified from Balestrieri et al. 2016.

1.2 The river otter in Ticino Valley

Ticino river is the largest tributary of the left bank of the Po river. Today, it is preserved by the Park of the Ticino Valley (Lombardy) , and the Natural Park of the Ticino Valley (Piedmont), covering 918 and 66 km^2, respectively. The river flows in the southern part of Lombardy (NW Italy), along a valley measuring 110 km long and 7 km wide, the overall length of the watercourse is 284 km, of which 91 km traveled upstream of Lake Maggiore. The river plain is characterized by intensive agriculture and anthropogenic activities, nevertheless natural reserves of riparian woodlands composed of english oak (*Quercus robur*), european white elm (*Ulmus laevis*) and field elm (*Ulmus minor*), european ashes (*Fraxinus excelsior/Fraxinus angustifolia*), and alluvial forest composed by alder (*Alnus glutinosa*), willows (*Salix alba* and *S. eleagnos*), and poplars (*Populus alba* and *P. nigra*) still cover long stretches of the river network. The ecological condition of the river is sufficiently good, excluding its southern part, where the concentration of hexachlorocyclohexanes (HCHs) exceeded the current limit according to 2000/60/EU Water Framework Directive (ARPA 2014).

In the early 20th century, the species in the area was considered common in lakes, rivers, swamps and rice fields (Ghigi, 1911; Cavazza, 1911), despite a decline in the provinces of Milan, Varese and Pavia (Bonelli and Moltoni, 1929). In the first half of the 20th century, the hunting activity of otter-hunter (“lontrari”) was so intense that in the first post-war years the species can be considered disappeared in several river sections, with the last individuals killed 1950 and 1974 in the territory of the actual Natural Park of the Ticino Valley (Piedmont). The last reliable sighting of river otter in the area through footprints dates back to 1980 at the ex-hunting reserve "S. Massimo ", in the municipalities of Gropello Cairoli and Garlasco, province of Pavia (Galeotti, 1981). In 1984-1985 the WWF national otter monitoring (Cassola, 1986), provided negative results (Bovio, 1986; Prigioni, 1986).

1.2.1 Reintroduction and the b-line question

The reintroduction of the otter in the Ticino valley has been proposed since the 1970s (Prigioni et al. 1979), this river had been identified as a potential reintroduction area in the *Action Plan for the Conservation of European Otters* (Macdonald e Mason, 1991). In 1978 started the "European Captive Reproduction Program" (Europäisches Erhaltungszucht Programm, EEP; Vogt, 1995) of the otter, with the collaboration of zoos and European breeding centers. At the beginning of the 1990s, almost 200 animals born and bred in captivity were available for reintroduction plans. Unfortunately, the analysis of mitochondrial DNA (Randi et al. 2001) of about 40 otters from which the EEP population originated, showed the evidence that some individuals of the Asiatic subspecies *Lutra lutra barang* were included. In addition, in the Italian breeding centers otters show a high level of in-breeding (Randi et al. 2001).

From the end of the 80s, the Natural Park of the Ticino Valley (Piedmont) has started a project of experimental reintroduction of the otter, creating the breeding enclosure "Centro lontra" in the locality Bosco Vedro, near Cameri (NO), a fence of 23600 m^2 which includes four pools of resurgence water for a total surface of 9200 m^2 (Montanari and Boffino, 2000). The first pair of otters, a two-year-old female from the Norfolk Wildlife Park in Witchingh in England, and a one-year-old male sold from the Zurich Zoo, was placed in the enclosure in March 1989. After the death of the male, a second individual, of two and a half years old coming from Norfolk, was introduced in March 1990. The couple has successfully reproduced since the first year (a female in 1990, two males in 1991 and two females in 1992, one male and one female in 1994). The couple of founders, both died in 1997, and have been replaced by a three-year-old male from the La Torbiera Wildlife Center (Agrate Conturbia) and a two-year-old female from the Caramanico Terme center (Pescara) daughter of one of the two males born at Bosco Vedro in 1991. The reintroduction took place on 22 August 1997, with the release of a male from the Torbiera and a young female daughter of the second pair of founders (Montanari and Boffino, 2000).

The loss of radio-collars after release doesn’t permitted to monitor the movements and behavior of the mustelids. Similar event had already occurred during the flooding episodes occurred in 1991 and 1993, when first a couple and a puppy, then a couple and two sub-adults, had escaped from the breeding center "La Fagiana” located in the Park of the Ticino Valley (Lombardy). In 2007, the carcass of a juvenile female was found at "La Fagiana", about 15 km downstream of the reintroduction site. In addition, some fingerprints were found at the end of the same year (Bellani A. pers. com.). In 2008, the otter was reported on a river stretch of about 5 km, which includes the breeding center of Cameri (Boffino, pers.com.). During a survey conducted between June and September 2008, the otter was recorded in only three sampling stations, approximately corresponding to 2.6 km section of the River Ticino, next to the release site (Prigioni et al. 2009). In 2010, an otter monitoring was performed on 35 km- of the Ticino River and otter spraints were found along a 7 km stretch of the river, mainly on canals and secondary arms. In late summer 2012, an adult otter was recorded about 30 km downstream the release site (Meriggi and Bellati, pers. Com.), while in winter 2013 two individuals were seen a further 10 km downstream (Cavalleroni, pers. com.). In March 2016 a young female was found dead by road-killing near Mulino di Limido in the Souther part of the Ticino River.

All these findings supported the presencein Lombardy Region on Ticino River of a small reintroduced otterpopulation (Fig 2, Balestrieri et al. 2016). Following Randi’s indications (Randi 2005; 2008), this population contains so-called B-line individuals, made up of haplotypes of non-European origin (Randi et al. 2005). This presence of external haplotypes is due to individuals from the reproduction program of otters in captivity of the “English Norfolk Otter Trust” concerning the European Breeding Program, in whose stock the presence of the subspecies *L. l. barang* from Southeast Asia has been ascertained. The presence in the European contest of external aplotypes has rised some conservation concerns by different authors.

1.3 The european catfish (Siluris glanis) case

Differents studies assert that in riverine ecosystem exotic species are the primary cause of decline in native fish populations mainly by predation, resource competition, interference with reproduction and/or the introduction of parasites and diseases (Taylor et al. 1984, Williamson 1996; Eby et al. 2006). Gozlan (2008; 2009) supports that exotic species are simply taking advantage of the biodiversity-loss process that is driven principally by habitat degradation and poor management practices. Non-native fish introductions have a long history in Europe (Copp et al. 2005), and one of the most popular and successful introduction has been that of the European catfish (*Silurus glanis*). *S. glanis* is the the world's third largest and the largest freshwater fish species in Europe (Stone 2007), indigenous to the European continent, *S. glanis* is native to Eastern Europe and Western Asia (Kinzelbach 1992), such as in the Danube and Volga river basins. It is now established in at least seven countries to the West and South of its native range (Elvira 2001) (Fig3.). The introductions of *S. glanis* throughout Europe occured mainly for aquaculture and angling (Copp et al. 2005), increasing popularity among fishermen because of the large size and relatively frequent capture, involved consequently in irregular introductions which are theoretically regulated by legislation in most European countries (Copp et al. 2005). Moreover european catfish have been introduced as a biocontrol agent for regulating cyprinids (Copp et al. 2009) because it is known to be a significant predator on native cyprinid stocks (Hickley and Chare 2004). In Italy European catfish have been introduced in the early 20th century for aquaculture, but the species was also introduced in ponds of private fishing reserves (Gandolfi and Giannini 1979; Boldrin and Rallo 1980). In rivers, the first report of this allochtonous species dates back to 1937 and is related to an individual caught in the river Adda (Manfredi, 1957) Its presence in rivers was initially considered occasional, linked to a load of fish imported from abroad. His acclimatization was observed with certainty in the late '70s (Gandolfi and Giannini, 1979). The potential impacts of *S. glanis* in its introduced range include disease transmission (Dezfuli et al. 1990), predation on native species and the modification of food web structure in some regions (Copp et al. 2009). Zerunian (2002) considered this species a serious threat for the indigenous fish populations, causing a reduction in the number of species and individuals. Despite these alarmist statements, *S. glanis* isn't a voracious predator, but an opportunistic scavanger, and as such it does not appear to present a remarkably great threat where introduced. Besides, Wysujack and Mehner (2005) reported that european catfish is a poor biomanipulator species. Furthermore, in most parts of Europe, where fish species have evolved in the presence of native piscivorous fishes (e.g. *E. lucius*, and *Perca fluviatilis*), the potential predatory impact of *S. glanis* is likely to be low (Hickley and Chare 2004). Finally, in light of the preponderance of non-native species in the diet of *S. glanis* in artificial water bodies (Carol 2007), predatory impact on native species is likely to be restricted to natural river stretches. The natural predators of european catfish include piscivorous such as pike (*Esox lucius),* great cormorants (*Phalacrocorax carbosinensis)* (Opačak et al. 2004) , and river otter (Lanszki & Körmendi 1996; Lanszki & Sallai 2006; Brzeziński et al 2006; Georgiev 2006; Miranda et al 2008; Gorgadze 2013).

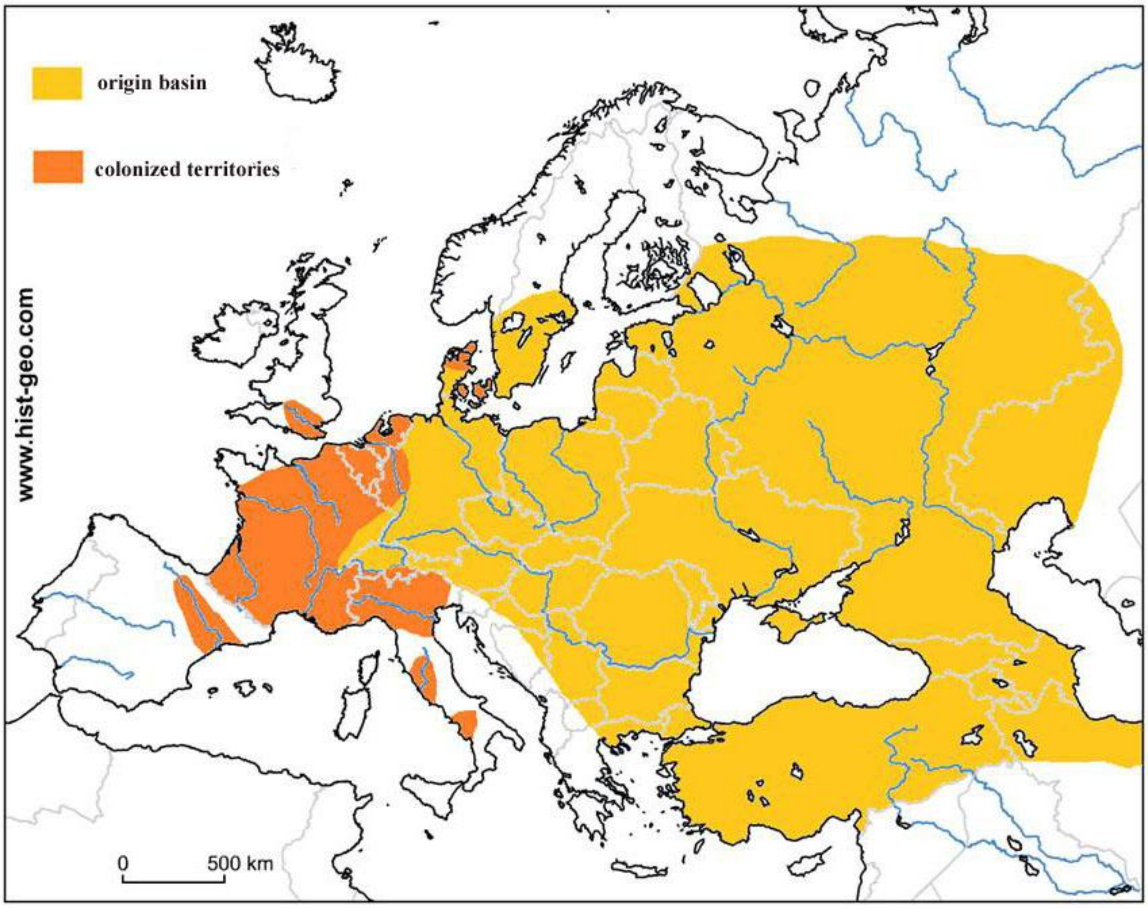

Fig3. *S. glanis* distribution in Europe; Guillaume et. al 2012

1.4 The problem of microplastics.

Nowadays environmental changes induced by human activities are unprecedented and represent a major threat to biodiversity (Brooks et al. 2002; Hanski 2011). Stream and rivers are essential components of landscapes and reflect the general condition of the territories where they flow through (Hynes, 1975; Allan, 2004). Consequently, their degree of pollution over large areas can be interpreted as a global indicator of the environmental health of the drained territories.

Plastic debris is considered a top environmental problem (UNEP, 2005; Gorycka, 2009), and is identified as an emerging issue that might affect human ability to conserve biological diversity in the near to medium-term future (Sutherland et al., 2010). In marine habitat large plastic items, such as discarded fishing rope and nets, commonly cause entanglement of invertebrates, birds, mammals and turtles (Carr, 1987; Fowler, 1987; Laist 1997; Gall and Thompson, 2015). Under environmental conditions, larger plastic items degrade to so-called microplastics, fragments typically smaller than 5 mm in diameter (Arthur et al. 2009). Their bioaccumulation potential is considered to increase with decreasing in size. In riverine environments, the dispersal and transport of microplastics is comparable to sediment transport. Flow velocity, water depth, bottom topography, river elevation and temporal variations, such as storms and floods, have a large influence on their distribution. The presence of water-flow-regulation structures, such as dams, greately affect fluxes of sediment and microplastic transport (Chanson, 2004). Recently the presence of microplastics in freshwater system in Europe have been found in Lake Geneva (Faure et al. 2012), in the Italian Lake Garda (Imhof et al. 2013), in the Austrian Danube river (Lechner et al. 2014), in the German Elbe, Mosel, Neckar, and Rhine rivers (Wagner et al. 2014), and in the UK Thames estuary (Sadri and Thompson, 2014).

Microplastic may be ingested by various organisms ranging from plankton and fish to birds and even mammals (Fig.4) and accumulate throughout the aquatic food web (Wright et al. 2013). Microplastics have been already reported in marine mammals, namely fur seals *Arctocephalus* spp. (Eriksson and Burton 2003), sea lion *Phocarctos hookeri* in sub Antarctic islands (McMahon et al. 1999) and harbour seals *Phoca vitulina* (Bravo Rebolledo et al. 2013), and fin whale *Balaenoptera physalus* (Fossi et al. 2014). The accumulation of debris in the digestive tract may cause a false sense of satiation leading to decreased food consumption (Ryan, 1988) and might facilitate the transport of chemical contaminants to organisms (Teuten et al. 2009). Microplastics could also have consequences for organisms at high trophic levels if any contaminants that are transferred have the potential for biomagnification (Teuten et al. 2009).

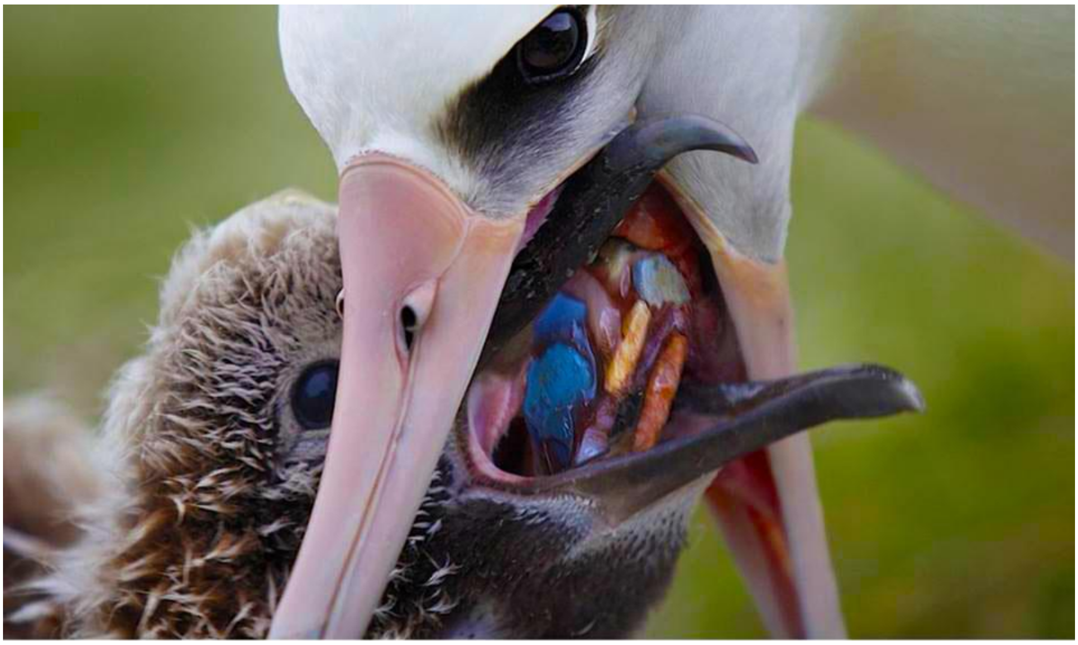

Fig4. A Laysan Albatross regurgitates plastic fragments to its chick. From www.albatrossthefilm.com

1.5 The southern otter Italian range. The Sila Massif.

The Sila massif located in Southern Apennines (Calabria region, South Italy), covering an area of ca. 2040 km^2 from the sea level up to 1928 m a.s.l. (Mount Botte Donato). This territory is a mountainous area, although without high summits. Because of its geographic position, this area has a high climatic variability with a particular subtropical climate, also known as the Mediterranean climate (Coscarelli and Caloiero, 2012). Corresponding to the altitudinal gradient, the vegetation contributes to a large habitat heterogeneity. From 0 to 800 m a.s.l., we find the "*Lauretum* belt", composed by Mediterranean maquis, Mediterranean pines and oak mixed forests. This is followed by the "*Castanetum* belt" from 800 to 1200 m a.s.l., which is composed by chestnut (*Castanea sativa*), oaks (*Quercus* sp.), maples (*Acer* sp.) and alders (*Alnus* sp.). At last, from 1200 m a.s.l. up to higher mountain reliefs, we find the "*Fagetum* belt", composed by *Fagus sylvatica, Abies alba Quercus cerris*, and *Pinus laricio*.

Following Prigioni et al 2006, two isolated residual populations occured in the Southern range of the Italian peninsula, a small nucleus in the Molise region, and a larger 'core' population in the river catchments of four regions: Campania, Apulia, Basilicata and Calabria. At the beginning of the twentieth century the Calabria region has been considered the southern limit of otter distribution in Italy (Cavazza 1911), although some historical reports of otter presence, referred to the18th and 19th centuries, was present in Sicily (reviewed by Tinelli and Tinelli 1986). During 1968–1972 otter was still widespread in the northern and central parts of Calabria region, especially in the freshwater system of the Sila Massif (Cagnolaro et al. 1975). Hunting activity, prohibited in 1977, over-fishing and the alteration of riparian habitats were thought to be the main causes of otter population reduction also in this area (Arcà 1986). The first national census carried out by the standard method (Reuther et al. 2000), following the monitoring of Macdonald and Mason (1982, 1983), showed an alarming reduction of the Italian otter range (Cassola, 1986; Fumagalli and Prigioni, 1993). In Calabria, the otter disappeared from the central and southern parts of the region on 13 waterbodies of the Sila Massif (Arcà 1986). Consequently Cassola (1986) indicated the threat of near extinction of the species in the region. Despite this, in 2002–2003, the otter was recorded on the rivers Savuto and Neto, flowing on opposite side of the Sila Massif, and on the River Crocchio, which flows southwards into the Ionian Sea (Fusillo et al. 2003). In 2008, a survey for otters promoted by the Sila National Park and Calabria Region confirmed the stable occurrence of the species on the River Savuto, which flows on the Western side of the Sila Massif (Balestrieri et al. 2008). A four year monitoring started in summer 2014 ascertained the presence of the otter in six rivers, (Amato, Lese, Neto, Savuto, Simeri and Tacina) for a total of 12 positive sampling stations (Gariano and Balestrieri, 2018).

1.6 Feeding behavior of Eurasian otter (Lutra lutra)

The Eurasian otter (*Lutra lutra*) in the aquatic community of European freshwaters and wetland systems is a top predator, considered an umbrella species, whose protection favors that of other species that use aquatic and riparian habitats (Bifolchi and Lodé 2005), and key species (Ruiz-Olmo and Jimenez, 2009; Clavero et al. 2010; Stevens et al. 2011), which contributes to sustaining the ecological balance of the riverine system (Chanin, 2003; Miranda et al. 2008), especially as regards the conservation and rehabilitation of ecosystem biotic integrity (Prenda et al. 2006). Consequently, many efforts were addressed to maintain and enhance the otter's populations in Europe which included habitat restoration and species re-introductions as well as studies of range development and diet analysis in numerous European countries (Jefferies et al. 1986, Bekker and Nolet 1990, Kožená et al. 1992, Prenda and Granado-Lorencio 1996, Copp and Roche 2003).

The semi-aquatic lifestyle of river otters is metabolically costly, with a daily consumption rate between 16% and 22% of their body mass. So otters are voracious hunter, but with the most restricted trophic niche in the mustelid family (Jedrzejewska et al. 2001, McDonald 2002) and, if the fish supply is not limiting, the otter is almost exclusively piscivorous (Ruiz-Olmo et al. 1989; Prigioni et al. 1991; Fusillo 2006; Remonti et al. 2008). In Europe the frequency of average occurrence of fish in feces is of 75% (Matos and Santos-Reis 2001; Clavero et al. 2003).

Moreover, fish have several more propitious peculiarity, specially the bottom-dwelling (benthonic) slow-moving species (Kruuk 2006), they usually present the higher biomass density in stable ecosystems (Beja 1995; Jiménez 2005; Ruiz-Olmo et al. 2003); they have a higher biomass per individual than crayfish or amphibians (Ruiz-Olmo et al. 2001), and they have a higher proportion of consumable parts. Moreover, fish provide higher energetic content (fish: 5.38–6.02 kJ/g wet weight; crayfish: 3.99–4.1 kJ/g wet weight; and amphibian: 2.87 – 3.42 kJ/g wet weight; Beja 1995; Ruiz-Olmo et al. 2007). Therefore, the diet is typically composed by cyprinids in eutrophic and calm waters, and by salmonids in oligotrophic and rapid waters (Mason and Macdonald 1986; Prigioni et al., 2006c).

Notwithstanding fish are the staple of otter diet, the high number of alternative food items found in otters' feces shows its plastic feeding behavior that tend to be more diverse in habitats with seasonally-variable environmental conditions (Ruiz-Olmo & Jiménez 2009).

Latitudinal gradient in otter diet was recently highlighted by Clavero et al. (2003), resulting in an increase of trophic diversity from northern to southern Europe. Nevertheless, more recently, Balestrieri et al. (2013) found no relationship between dietary breadth and latitude, suggesting that habitat-related variations in fish assemblage play a major role in shaping otter diet.

In Mediterranean regions, climatic fluctuations, such as unpredictability of precipitation and temperature regimes, influencing prey availability for otters, are reported as the main factor increasing otter trophic diversity in southern Europe (Ruiz-Olmo et al. 2001; Clavero et al. 2003). Moreover, hydraulic structures (e.g. dikes, weirs and dams), over-fishing, modify the ecological interactions of riverine species (Nilsson et al. 2005; Perez-Quintero 2007; Wang et al. 2011). The same factors cause the decrease in fish availability during dry periods that forces otters to extend the spectrum of its prey (Delibes and Adrián 1987; Beja 1996; Román 2011, Ruiz-Olmo et al. 2001), toward alternative prey taxa such as amphibians, reptile, crabs, crayfish, and insects (Mason & Macdonald 1986; Jedrzejewska et al. 2001, Clavero et al. 2003, Smiroldo et al. 2009). Following reptiles and crustaceans, amphibians are the most important alternative food for this mustelid, their presence in the otter's diet is variable and on average it is in the order of 10%. In Russia (Grigorev and Egorov, 1969) and in certain areas of Sweden (Erlinge 1969), the percentage rises to 45-50%, especially in coincidence with the hibernation period of frogs. Meanwhile, in Mediterranean area, such as South Italy, amphibians play a major role as alternative prey (Remonti et al. 2008, 2009; Smiroldo et al. 2009), particularly during spring when anurans aggregate in high densities to spawn (Clavero et al. 2005). Otters prefers selection of frogs, rather than toads (Weber 1990; Sidorovich and Pikulik 1997; Clavero et al. 2005), *Bufonidae* are harder to manipulate for the poisonous glands on their skin, which are a potential source of injury, when the mustelid trying to reach the flesh spend long time handling them to avoid a possible trauma (Lizana and Pérez Mellado 1990; Ruiz-Olmo 1995). This feeding behaviour on *bufonidae* has been described in Portugal (Beja, 1996), Spain (Lizana & Pérez Mellado 1990), Belarus (Sidorovich and Pikulik, 1997) and Finland (Sulkava 1996). Furthermore, cases of predation on *bufonidae* from mammal are known such as polecat (*Mustela putorius*), shrews (*Sorex araneus*), and the wild boar (*Sus scrofa*).

In the last two decades, newt remains have been recovered in the stomachs of otter carcasses both in SW England (*Triturus cristatus*; Britton et al. 2006) and Hungary (*Lissotriton vulgaris*; Lanszky et al. 2015), while newts have been recorded in otter spraints in Wales (*Lissotriton helveticus*; Parry et al. 2015) and the Czech-Moravian Highlands (Poledník et al. 2007). Furthermore, direct observations of otters preying newts have been recorded such as *Triturus cristatus* in North Jutland (Fig.5) (Bringsøe and Nørgaard, 2018) and *Pleurodeles waltl* in South Spain (Cogălniceanu et al.

2010). This feeding performance suggests an opurtunistic behavior of the mustelid which diet tends to change depending on the abundance of the different prey types (Ottino and Giller 2004).

Fig.5 Eurasian otter has caught a *Triturus cristatus. Photos by Jens Nørgaard* (Henrik Bringsøe,and Jens Nørgaard 2018)

1.7 Study aims and thesis structure

Otter distribution in Italy is currently changing, in the North there are otters coming from the nearest Austrian ans Slovenian populations and the bigger South core area composed by Basilicata, Campania, Apulia and Calabria shows slightly positive trends.

The most emblematic example of the efforts for the recovery of this mustelid is represented by the area of the Ticino River, where a reintroduction project was attempted. The first aim of this thesis is to verify the status and the actual distribution of the otter population on the Ticino River by a survay along all the sublacual portion of the river (110 km).Chapter 2 describes the results of the otter monitoring on Ticino River together with the analysis of the diet obtained by the undigested remains in stools. Two main results emerged: the presence of *S. glanis* within the preyed fish species and the presence of microplastics in otter stools. *S. glanis* was never recorded in previous diet studies performed on Italian otter feces. Undigested remain of this species were studied by means of 28 specimens of *S. glanis* taken from the same area, K index that consent to established the size of the preyed fish from a morphometric analysis was established, thus opening new perspectives for more detailed studies on otter's diet and on the efficiency of *S. glanis* predation by otters (Chapter 3).

Otter distribution in Sila Massif (South Italy) was studied to compare the North and South extremes of the otter distribution in Italy, even if the otter range in Italy is largely far from being continuous. An important consumption of amphibians in the otter diet on Sila Massif appeared as the most important results and it was related to the relative abundance of fish and amphibians in the same area (Chapter 4). In the mean time, the appearance of unknown materials in otter stools let us to execute an Attenuated Total Reflectance (ATR-FT-IR) spectroscopy, as in the sace of microplastics, to identify a possible ingestion of frog spawn by the mustelid (Chapter 4).

Actually, to my knowledge an exhaustive work on the recognition of amphibian species and genus, were never been tempted. For this purpose, a detailed osteological study of amphibian remains in otter stools was performed by a collaboration with the Deptartment of Earth Sciences of the University of Turin (Chapter 5). In addition a literature review was realized in order to analysed the amphibian role in otter diet (Chapter 5). Finally, the amphibian monitoring, performed in the Sila Massif to completion of the otter monitoring, was analysed with the aim of comparing the recorded amphibians to the otter presence. Otter were recorded in near half of the stations, thus it was possible to test the impact of otter presence on the amphibian community (Chapter 6).

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2. Anthropogenically altered trophic webs: alien catfish and microplastics in the diet of Eurasian otters

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Anthropogenically altered trophic webs: alien catfish and microplastics in the diet of Eurasian otters.

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ABSTRACT

With the aim of examining how Eurasian otters (*Lutra lutra*) face human-mediated environmental alterations, we assessed their diet by spraint analysis on the River Ticino (NW Italy), where this mustelid has been reintroduced in 1997. From March 2016 to March 2017, a total of 101 spraints was found in 50% of 32 sampling stations (mean length ± SD = 567 ± 263 m). Fish formed the bulk of otter diet (95% of the estimated mean percent volume, mV%). Cyprinids were the most preyed fish (mV% = 44.9), followed by European catfish *Silurus glanis* (mV% = 24.9%) and eel *Anguilla anguilla* (mV% = 8.5). Introduced European catfish is an invasive species, which can deeply alter the composition and structure of local fish communities and accumulate large amounts of metals and pollutants through the trophic chain. We also recorded for the first time microplastic particles (< 5 mm) in otter spraints. Suspected particles were analysed by Fourier transform infrared (FTIR) spectroscopy and two polymer types were identified: polyethylene terephthalate (PET) and polyamide (PA). Although otters showed to be able to adapt to anthropogenic changes, these results point out new potential threats to otter conservation and ask for further studies.

Keywords: Eurasian otter; *Lutra lutra*; marking activity; microplastics, *Silurus glanis*; endangered species.

INTRODUCTION

The current scale and rate of human-induced environmental change is unprecedented and represents a major threat to biodiversity (Brooks et al. 2002; Hanski 2011). Economic activities affect species abundance and distribution through landscape modifications which alter ecosystem properties and functions (Foley et al. 2005), including productivity (Haberl et al. 2007) and food availability (Muhly et al. 2013). Human settlements are still expanding rapidly, forming, together with agricultural cropland, large areas of modified landscapes where resource subsidies are regulated by human activities (Newsome et al. 2014).

In such altered environments, anthropogenic resources - e.g., livestock, garbage, pet food, road-kills, and synanthropic rodents and birds (Yirga et al. 2012) - are increasingly reported as a major factors affecting predator–prey relationships, particularly for terrestrial mammalian predators (Contesse et al. 2004; Newsome et al. 2014; Wierzbowska et al. 2017).

A further human-driven food resource for native predators is represented by introduced invasive species (Rodriguez 2006; Schlaepfer et al. 2011; Barbar et al. 2016), the spreading of which have been reported to be enhanced by altered ecosystems (Moyle 1986; Crooks et al. 2011). 'New' food resources may sometimes have beneficial effects on predator abundance (Pintor and Byers 2015), particularly on the populations of food-limited predators, and enhance the survival of species of conservationist interest (Beja 1996). As an example, introduced brown rats (*Rattus norvegicus*) and especially rabbits (*Oryctolagus cuniculus*) may have played a major role in the survival (in the 1950s) and ongoing recovery of polecats (*Mustela putorius*) in Britain (Birks 2015; but see Lambertucci et al. 2009 about the potential threat posed by exotic prey to scavenging birds).

While polecats—together with other mustelids (stone marten Martes foina and Eurasian badger *Meles meles*) and several carnivore mammals (reviewed by Bateman and Fleming 2012)—currently live successfully in farmland and suburbs, relying on pet food and synanthropic prey (Birks 2015), Eurasian otters (*Lutra lutra*) generally avoid human settlements and their occurrence in man-altered habitats results from the ongoing encroachment of urban settlements and agricultural land upon wilderness areas (see Radeloff et al. 2005).

Accordingly, otters usually do not use anthropogenic food, mainly relying on fish and semiaquatic prey (Kruuk 2006). Since Roman times, however, and increasingly in the last century, freshwater fish assemblages have been deeply altered by the introduction of a plethora of fish species (Elvira 2001; Leprieur et al. 2009; Strayer 2010), leading to taxonomic homogenisation of fish assemblages (Marr et al. 2010). The Near Threatened Eurasian otter (IUCN Red List; Roos et al. 2015) may thus take advantage of the still increasing availability of exotic food, particularly where native fish species have been depleted by overfishing and damming. Notwithstanding, otter predation on alien fish seems, on average, negligible throughout its European range (Balestrieri et al. 2013), with a few exceptions reported for the Mediterranean area, particularly in the Iberian peninsula and heavily human-altered areas (Balestrieri et al. 2013).

In Italy, otter populations declined throughout the twentieth century, but are currently recovering in southern regions, the core Italian otter range, and the north-east of the peninsula, thanks to otters expanding from neighbouring Austria and Slovenia (Balestrieri et al. 2016). In northern Italy, the otter was extirpated in the 1980s (Prigioni 1986; Prigioni et al. 2007). On the River Ticino (Lombardy region), a reintroduction was attempted in 1997, by releasing 1–2 otter pairs (Montanari and Boffino 2000; Balestrieri et al. 2016). Unexpectedly, the reintroduction was successful, and 10 years, later the otter still occurred in the release area (Prigioni et al. 2009); in 2010, its presence was confirmed on a 7-km-long stretch of the river, with a few direct observations 30–40 km downstream (Balestrieri et al. 2016).

Although the plain of the River Ticino includes the largest and best conserved riparian woods of northern Italy, this watercourse crosses an intensively cultivated and urbanised area and ca. 50% of its fish species is non-native (GRAIA 1999). Moreover, in the last decade, introduced European catfish (*Silurus glanis*) has rapidly spread into the river, with dramatic, although still insufficiently known, consequences on the fish community (GRAIA 2007).

Hence, the question is how otters can thrive in this wildlife-urban interface and to which extent they might be able to moderate human multifaceted impacts (e.g., Kranz and Toman 2000; Kloskowski et al. 2013). As their diet has been poorly investigated to date (Prigioni et al. 2009), we aimed to assess the effects of human-mediated prey availability on otter feeding behaviour through the analysis of undisputable otter faeces. In the heavily disturbed reintroduction area, we expected otters to exploit the most widespread fish species, regardless of their origin (native vs. alien species). Faecal samples were searched for on the whole Italian stretch of the river, providing additional information on the distribution of the otter 20 years after its reintroduction.

MATERIALS AND METHODS

Study area

The study was carried out on the Italian stretch of the River Ticino (110 km), between the southern edge of Lake Maggiore and its confluence in the median course of the River Po (Lombardy region, N Italy; Fig. 1). The river crosses an intensively cultivated and urbanised plain; nonetheless, well-

conserved riparian habitats still border long stretches of the watercourse. Riparian woods consist of English oak (*Quercus robur*), elms (*Ulmus laevis* and *U. minor*) and ashes (*Fraxinus excelsior* and *F. angustifolia*). Alluvial forests, consisting of alder (*Alnus glutinosa*), willows (*Salix alba* and *S. eleagnos*) and poplars (*Populus alba* and *P. nigra*) are still widespread inside the weave of meanders, streams, canals and oxbow lakes, which characterise the downstream stretch of the river. The climate is temperate with a mean annual temperature of ca. 13 °C. Mean annual rainfall decreases from north (1200 mm) to south (700 mm).
The ecological status of the river is "good", except for the stretch downstream Pavia, where the concentration of hexachlorocyclohexanes (HCHs) exceeded the current limit values according to Community Directives (Dotti et al. 2014). The River Ticino valley is currently protected by the Park of the Ticino Valley (Lombardy), covering 918 km^2, and the Natural Park of the Ticino Valley (Piedmont), 66 km^2.

Surveys for otter faeces

From March 2016 to March 2017, a total of 32 station sampling stations (mean length ± SD = 567 ± 263 m; min–max: 100–1220, depending on bank accessibility) were surveyed for otter faeces ("spraints") following the "Standard Method" recommended by the IUCN/SSC Otter Specialist Group (Reuther et al. 2000). During each survey, all shoreline areas were searched by walking on both riversides and around small islands and bars looking for typical otter sprainting sites (e.g. large stones, bridges, pool banks, confluences; Macdonald and Mason 1983; Prigioni 1997). A sprainting site was identified as a place with spraints that were at least 1 m from the other ones (Kruuk et al. 1986). Each station was surveyed, on average 2 ± 1.5 times (min–max: 1–9), for a total of 62 surveys. Spraints were stored individually in silver paper and labelled with an univocal code. Otter marking activity was expressed as both number of spraints per 100 m and, for sampling stations surveyed twice or more, percentage of surveys positive for otter presence [P% = (number of positive surveys/total number of surveys) × 100)].

Diet analysis

Before the analyses, each sample was soaked for 24 h in a solution of distilled water and hydrogen peroxide, then washed through a sieve with a 0.5-mm-wide mesh, dried in oven, and observed under a binocular microscope (Leica Wild M3Z 6.5-40x, UK). Fish remains were identified from their vertebrae, jawbones and scales, using personal collections and the keys of different authors

(Webb 1976; Wise 1980; Camby et al. 1984; Prigioni 1997). Amphibians were identified by the keys of Di Palma and Massa (1981), whilst telson, chelae and thoracopods were the main diagnostic features for crustaceans. Following Prigioni et al. (1991), results were then expressed as percent frequency of occurrence (F% = (number of spraints containing a specific food items/total number of examined spraints) × 100), percent relative frequency of occurrence (rF% = (number of occurrences of an item/total number of items) × 100), estimated percent volume (V% = total estimated volume of each food item as ingested/number of spraints containing that item) and percent mean volume (mV% = total estimated volume of each food item as ingested/total number of examined spraints), which outlines the proportional contribution of each food item to the overall diet (Kruuk and Parish 1981) .

For each sample the minimum number of individuals of each kind of prey was estimated by the number and position (left–right) of diagnostic hard parts (as mouth bones for fish, illions for amphibians). When no diagnostic part was found, the remains of a prey were considered to belong to a single individual. The weight of preyed fish was assessed by comparing the size of the jawbones found in otter spraints to a personal reference collection of jawbones collected from fish of known size. A constant weight was assigned to the other prey items: insects 1 g, amphibians 30 g, reptiles and crustaceans 50 g, birds 100 g.

Trophic niche breadth was estimated by standardised Levins' index $B = 1/(R\ 2\ \Sigma p\ i\ )$ (Feinsinger et al. 1981), using mV% (p i) and grouping data in R = 6 food categories (Centrarchidae, Anguillidae, Salmonidae, Siluridae, Cyprinidae, other items, the latter including all items with mV% < 2). Data were then grouped seasonally, distinguishing a warm (April–September, N = 31) and a cold (October–March, N = 65) period and raw frequency data were compared using the chi-squared test (χ^2). The relationship between catfish mV% and both the distance of the sampling station from Lake Maggiore and richness of the fish community (as assessed for 5-km-long stretches of the river; GRAIA 1999; Prigioni and Balestrieri 2011) was tested by Spearman's correlation.

Analysis of foreign materials

Materials of unknown composition (mainly fragments, pellets, filaments in the size range of 0.5–5 mm) were isolated, photographed by a stereo microscope LEICA EZ4 D with Integrated LED illumination and Digital 3 MP Camera, and their colour, shape and dimension were recorded. As soon as plastic-like fragments were found, in order to exclude laboratory airborne contamination, 3–5 glass Petri dishes were put on the workbench near the operator (McCormick et al. 2014).

As visual inspection is insufficient and tends to overestimate microplastic abundance, infrared spectra of 24 samples of suspected plastic were obtained using attenuated total reflectance (ATR-FTIR) spectroscopy to identify polymer types (Mintenig et al. 2017). The ATR technique allows the simple, reproducible and non-destructive collection of IR spectra of small samples without sample preparation. A Perkin Elmer Universal ATR Sampling Accessory, consisting of a diamond crystal, was used. After each analysis, the crystal was cleaned with acetone. Spectra were collected using a Perkin Elmer FTIR Spectrometer Spectrum One (PerkinElmer Waltham, MA, USA). Analyses were performed using a spectral resolution of 4^{-1} cm and 32 scans in the range 4000–650 cm^{-1}.
Spectra were compared to a spectral customised library including the spectra of all common polymers, natural materials and lab products used for purification. Only polymers matching reference spectra ≥ 60% were accepted (Lusher et al. 2013).

RESULTS

A total of 101 spraints was collected in 41 marking sites from 16 sampling stations. Otter signs were distributed along the whole watercourse (50% of sampling stations were positive for otter presence; Fig. 1), including some small tributaries and canals. Average marking intensity was 0.20 spraints per 100 m of watercourse and 0.1 marking sites per 100 m, while the mean percentage of positive surveys was 54% ± 35.6%.
Fish formed the bulk of otter diet (F% = 97.9, mV% = 95.0; Table 1). Most preyed fish were cyprinids (F% = 75.0, mV% = 44.9; Fig. 2) - particularly barbel *Barbus sp.* (F% = 45,8), chub *Squalius cephalus* (F% =28.1), Western vairone *Telestes soufia* (F% = 17.7) and bleak *Alburnus alborella* (F% = 29.2) - followed by introduced European catfish (F% = 45.8, mV% = 21.8) and eel *Anguilla anguilla* (F% = 24.0, mV% = 8.5). Food items other than fish accounted for 1–2% of the overall diet each (Fig. 2). Eel and tench *Tinca tinca* were preyed upon more frequently in the warm season (eel: F% = 48.4 and 12.3, respectively; χ^2 =15.0, $P < 0.001$; tench: F% = 29.0 and 3.1, respectively; χ^2 =13.9, $P < 0.001$), while the remains of the Western vairone prevailed in the cold season (F% = 3.2 and 24.6; χ^2 = 6.6, $P = 0.01$). The mV% of catfish was inversely related to the richness of the fish community (rho = − 0.67, P = 0.007, N = 15), which increased with the distance of the sampling station from Lake Maggiore (rho = 0.72, P = 0.002, N = 15). Yearly trophic niche breadth (B) was 0.45.
Most of the particles of unknown composition, found in otter spraints, looked broadly spherical, 500–3500 μm in diameter, with iridescent or translucent amber colorations (e.g. Fig. 3 d, e h, l, n),

while some, by their aspect and dimension, looked like microplastics (e.g. Fig. 3 b, c and g). Two samples were confirmed of anthropogenic origin. The Tic-B spectrum (Figs. 3b and 4) showed C–H stretching bands at 2869 and 2908 cm^{-1}, a strong C=O band at 1712 cm^{-1} and C–OC bands at 1242 and 1094 cm^{-1}, typical of polyethylene terephthalate (PET; correlation = 97%). The Tic-C spectrum (Figs. 3c and 4) showed a strong N–H stretching at 3296 and 3088 cm^{-1} and C=O and C–N stretchings at, respectively, 1633 and 1537 cm^{-1}, suggesting the presence of nylon polyamide (PA; correlation = 65%). A sample of fibres (Fig. 3g) showed the typical bands of wool proteins (correlation = 70%). All other items (Fig. 3) showed ATR/FTIR protein-like spectra of natural origin. In fact, in the spectra are evident stretching bands attributable to N–H (3300 and 3070 cm^{-1}), symmetric and asymmetric H-Csp^3 (2940 and 2870 cm^{-1}) and amidic C=O (1640 cm^{-1}) bands. Visually, after having been treated with diluted hydrogen peroxide and over-dried, the dimension, shape and colour of some were very similar to amphibian eggs, which we took from a *Xenopus leavis* breeding as reference material. For comparison, ATR/FTIR spectra of amphibian, fish and crustacean eggs were analysed and resulted very similar among them (similarity > 80%; Fig. 5), and to samples from otter spraints (similarity = 79%). The remaining materials of probable anthropogenic source but natural origin were cellulose and cotton (ATR/FTIR spectra not shown).

DISCUSSION

Improving our understanding of human impacts on food webs is especially important for the conservation management of top predators both in areas where they are still declining and, just as importantly, in human-dominated landscapes where they are recovering either spontaneously or following reintroductions (Dorresteijn et al. 2015). We investigated the diet of Eurasian otters 20 years after their reintroduction on the River Ticino with the aim of assessing the impact of human-mediated changes in the composition and richness of the fish community on its diet. Particularly, we expected to observe the effects of the recent spread of European catfish, which, in spite of several attempts to control its population abundance by selective electro-fishing, in the last decade has colonised the whole watercourse, deeply altering the native fish community (e.g. GRAIA 2007). Otter diet faithfully mirrored the ongoing invasion, catfish being otter main prey in terms of frequency of use. Intensive predation can provide biotic resistance to invasion (de Rivera et al. 2005; Tetzlaff et al. 2010) and predation risk can deeply affect prey behaviour and reproductive output (Lima and Dill 1990; Lima 2002); otters, mainly preying upon individuals < 2 years (i.e.

< 50 cm in length; GRAIA 2007), currently may represent one of the few native predators able to lower catfish reproductive potential in the area of introduction.

Otter diet also agreed with that of the catfish itself (GRAIA 2007), which, as the mustelid, is a top predator of aquatic habitats, suggesting that otters preyed opportunistically on the currently most widespread fish species (bleak, barbel and chub). Consistently, monitoring of dam fish ladders by video cameras confirmed that barbel, chub and Western vairone are among the commonest fish species of the river (Puzzi et al. 2014).

In contrast, in 2008, otter relied mainly on Eurasian minnow *Phoxinus phoxinus*, South European nase *Protochondrostoma genei*, trout Salmo trutta and perch *Perca fluviatilis* (Prigioni et al. 2009). It is hard to compare the two studies, as fish availability on the few-kilometre-long stretch where otter occurred in 2008 (Prigioni et al. 2009) may have been quite different from the average for the whole river. Nonetheless, available fish data support the hypothesis that the variation in otter diet may depend, at least in part, on changes in the fish community that occurred in the last decade (GRAIA 1999; Puzzi et al. 2014; Consorzio del Ticino,and Parco Ticino 2016).

In the Mediterranean basin, crustaceans and amphibians often provide a major contribution to otter diet (Clavero et al. 2003), even during the winter hibernation phase (Prigioni et al. 2006a; Smiroldo et al. 2009), while in the study area, their frequencies of occurrence in otter spraints were negligible (1.0% and 4.2%, respectively), also with respect to the previous investigation (F% = 8.3 and 11.1, respectively; Prigioni et al. 2009). As otters prey on alternative-to-fish prey when fish become scarce (Remonti et al. 2008), these results suggest that changes in the composition of the fish community did not lower their overall availability to otters. Accordingly, catfish seemed to play a valuable role as an autochthonous fish substitute in river stretches hosting fish communities impoverished by damming, in the upstream section, overfishing and the spreading of catfish itself (Puzzi et al. 2007, 2014).

The analyses of foreign materials revealed the occurrence of microplastic fibres in otter spraints. Currently, among the multiple human pressures, the accumulation of plastic debris is a serious environmental problem for its negative biological impacts on aquatic ecosystems (Derraik 2002; Andrady 2011; Cole et al. 2011; Eerkes-Medranoa et al. 2015). Durability, unsustainable use and inappropriate waste management cause an extensive accumulation of plastics in natural habitats (Barnes et al. 2009). Under environmental conditions, plastic materials degrade to so-called microplastics, fragments typically smaller than 5 mm in diameter (Arthur et al. 2009), increasing their potential for bioaccumulation. Deliberately produced microplastics (e.g. those added in cosmetics) and microfibers released from synthetic tissues contribute to the overall environmental burden (Browne et al. 2011).

Because of their small size, microplastics can be mistaken for food and can be ingested by a variety of organisms (Ivar Do Sul and Costa 2014), ranging from plankton (Setälä et al. 2014) to fish (Lusher et al. 2013; Foekema et al. 2013). There is increasing evidence that microplastics can be transferred between trophic levels of food webs and, particularly, in aquatic environments, the highest trophic levels ingest microplastics via fish prey (Eriksson and Burton 2003; Tanaka et al. 2013). While microplastics have been already reported in marine mammals, namely fur seals *Arctocephalus spp.* (Eriksson and Burton 2003) and sea lion *Phocarctos hookeri* in subAntarctic islands (McMahon et al. 1999) and, possibly, basking shark *Cetorhinus maximus* and fin whale *Balaenoptera physalus* (Fossi et al. 2014), to our knowledge, this is the first record of microplastics in a top predator of freshwater ecosystems. Otters probably ingested microplastics through predation on benthophage cyprinids, such as barbel and chub. Accordingly, the otter has been reported to be at risk of microplastic accumulation from lower trophic levels in Irish freshwaters (Mahon et al. 2017). For mammals as large as otters, microplastic toxicity is likely to epend mainly on their associations with chemicals, particularly metals and persistent organic pollutants (POPs), which can reach concentrations even higher than those in sediments (Engler 2012; Bakir et al. 2012; Holmes et al. 2014).

Notwithstanding habitat alterations, with respect to previous studies (Prigioni et al. 2009; Balestrieri et al. 2016), the research has drawn an encouraging picture of the distribution of the otters in the Ticino valley, with a sharp increase in the length of the river stretch used by the mustelid. Marking intensity was still low with respect to the core area of the Italian otter range (3.17 spraints/100 m; Prigioni et al. 2005), suggesting that the whole population should consist of a few individuals (Prigioni et al. 2006b), although we cannot exclude that the hydrological complexity of the study area did not prevent us from assessing marking intensity effectively.

The otter showed to be capable of adapting to anthropogenic changes in the availability of food resources of the reintroduction area, relying on most widespread fish prey, included non-native catfish. Regrettably, being top predators, catfish may accumulate large amounts of metals and persistent organic pollutants (POPs), mainly through trophic–chain biomagnification (Gobas et al. 1993), and the actual transfer of pollutants from fish to their predators has been demonstrated (Volta et al. 2009). In the River Po basin, the levels of mercury in *S. glanis* have been reported to exceed the threshold level of 0.5 ppm in 18% of samples, whilst the concentrations of cadmium, lead, arsenic and chromium were below permitted maximum levels (Squadrone et al. 2012).

We should thus be aware that diet shift from native cyprinids to catfish may threaten the conservation of the reintroduced otter population. Great attention should be posed to water quality downstream of Lake Maggiore, which suffers a severe mercury and DDT contamination due to the past activity of a chemical plant (C.N.R.-I.S.E. 2016) and close to the confluence into the River Po, where hexachlorocyclohexanes (HCHs) exceed EU limits (Dotti et al. 2014).

Microplastics in freshwaters have been under-investigated compared to marine systems (reviewed by Anderson et al. 2016). As the record of microplastics in otter diet was an unplanned, fortuitous event, it is likely that they were underestimated and their presence in otter spraints may represent the “tip of the iceberg” of a wider environmental contamination. Consistently, microplastics accumulate in freshwaters flowing in areas of relatively high human density (Eriksen et al. 2013; Castañeda et al. 2014; Yonkos et al. 2014). Under these premises, we argue that otter conservation urgently requires further investigations on the abundance of both otter population and microplastics in freshwater sediments.

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Tab.1 Overall diet of reintroduced otters as assessed by the analysis of 96 spraints collected on the River Ticino (N Italy).

Food items	F%	rF%	V%	mV%
Insects				
Coleoptera	1	0.4	1	0.01
Crustaceans	1.0	0.4	10.0	0.1
Fish	97.9	36.3	97.0	95.0
Centrarchidae				
Perca fluviatilis	4.2	1.5	45	1.9
Anguillidae				
Anguilla anguilla	24.0	8.9	35.7	8.5
Salmonidae				
Salmo trutta	5.2	1.9	23	1.2
Siluridae				
Silurus glanis	45.8	17	47.6	21.8
Cyprinidae	75.0	27.8	59.9	44.9
Undetermined cyprinids	3.1	1.2	26.7	0.8
Barbus spp.	45.8	17	36.9	16.9
Protochondrostoma genei	2.1	0.8	25.0	0.5
Squalius cephalus	28.1	10.4	42.6	12
Telestes soufia	17.7	6.6	36.5	6.5
Tinca tinca	11.5	4.2	21.4	2.4
Alburnus alborella	29.2	10.8	19.8	5.8
Undetermined fish	41.7	15.4	39.9	16.6
Amphibians				
Rana sp.	4.2	1.5	41.3	1.7
Reptiles	1	0.4	50.0	0.5
Birds	2.1	0.8	80.0	1.7
Mammals	1	0.4	100	1
Fruit	1	0.4	5	0.1

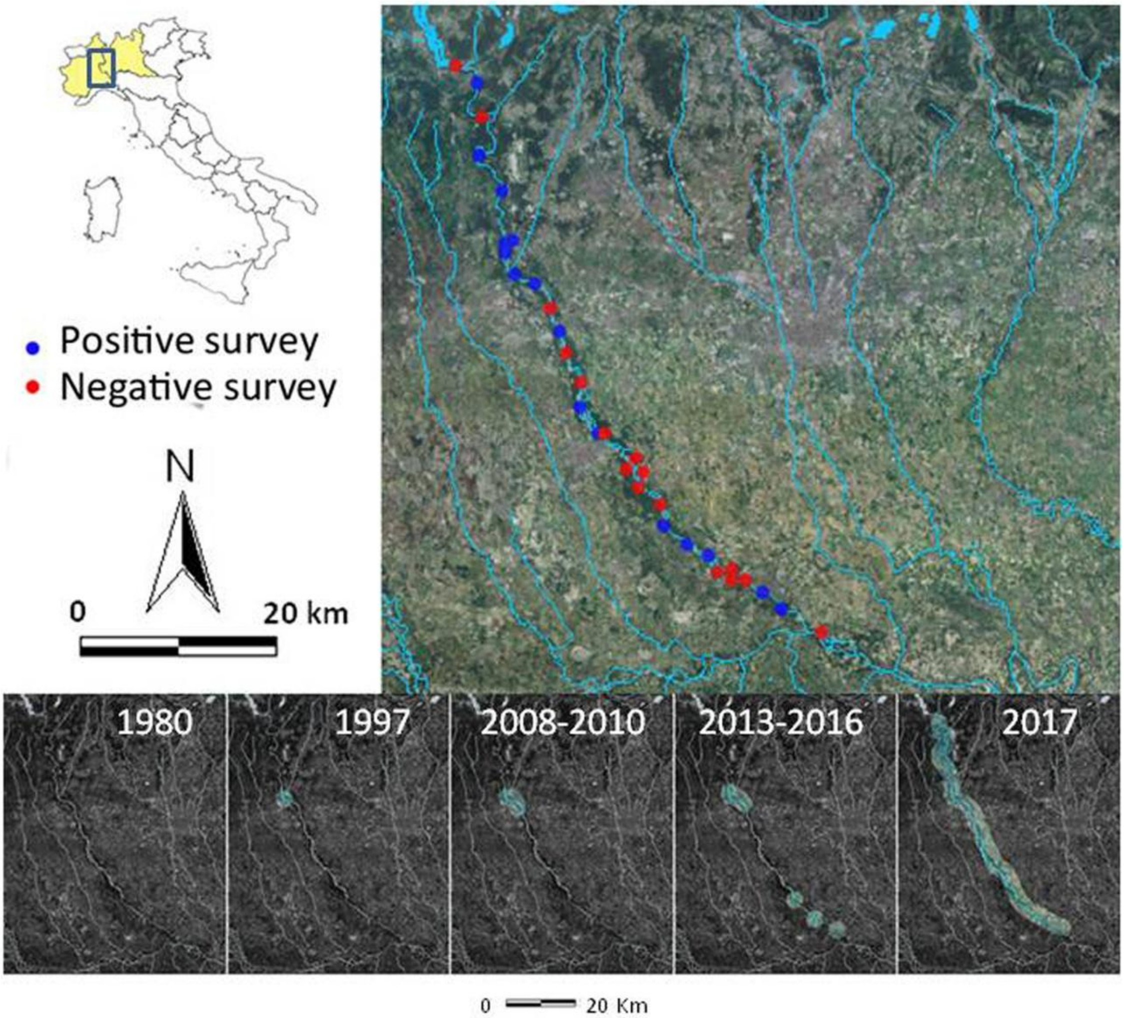

Fig.1 Sampling stations positive and negative for otter spraints on the River Ticino (03/2016 – 03/2017)
Time range evolution of the otter in the study area

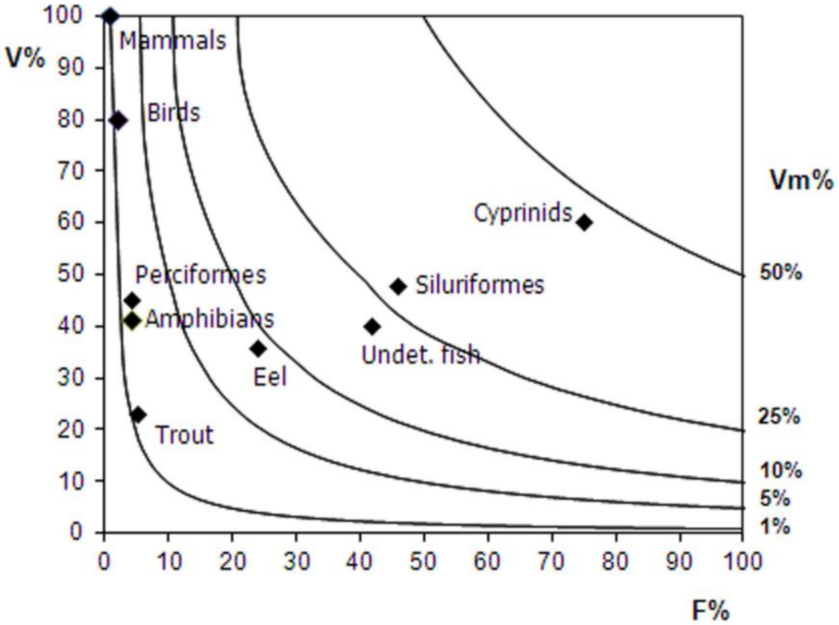

Fig.2 Estimated volume (V%) of the main food categories, whenever eaten, *vs*. their frequency of occurrence (F%) for the overall diet of the otter on the river Ticino. Isopleths connect points of equal percent mean volume in the diet (mV%, see methods).

Fig.3 Materials found in otter spraints and suspected to be microplastics.

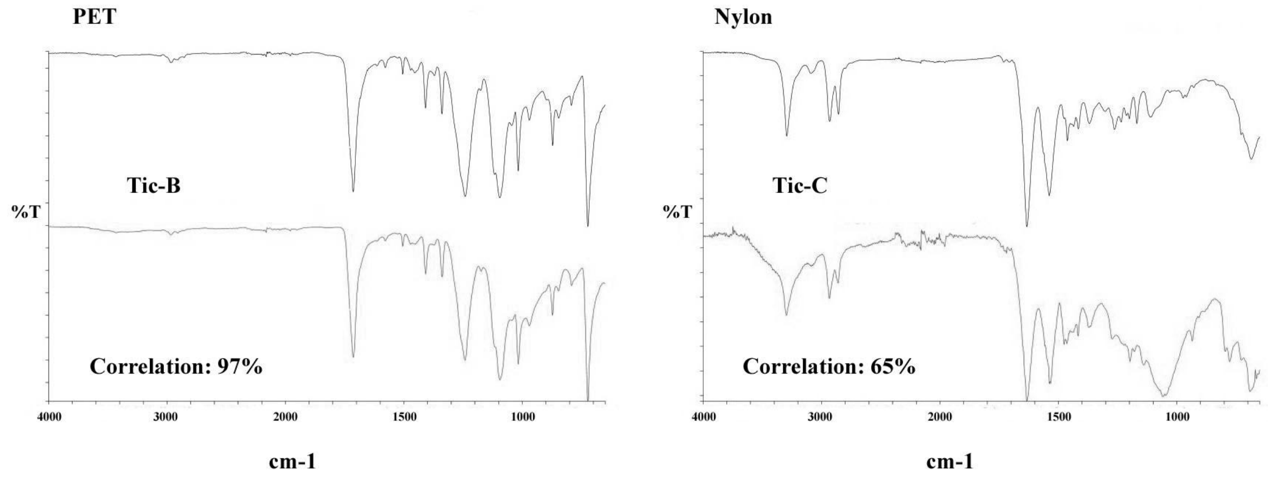

Fig.4 Spectra comparing plastic polymers (%T = trasmittance; cm-1 = wave number) to tangled filaments found in otter spraints on the River Ticino: TIC – B and TIC – C refer to images b and c in fig. 4.

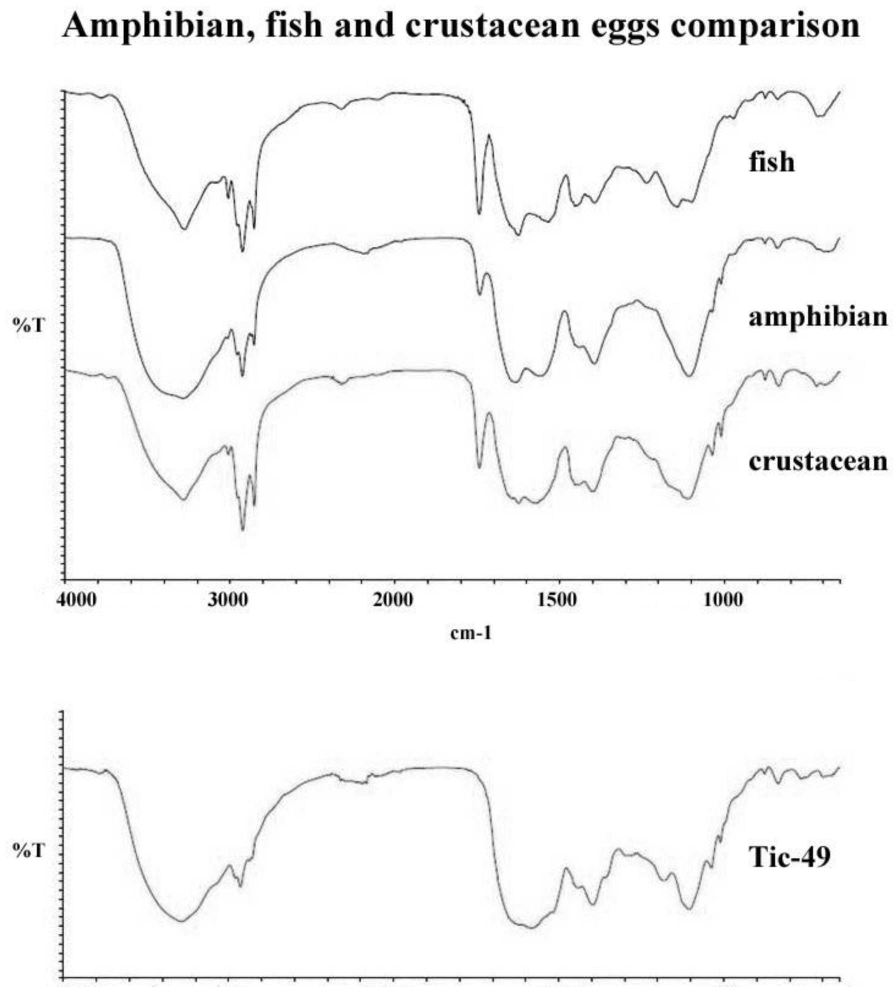

Fig.5 Spectra comparing amphibian, fish and crustacean eggs to materials found in otter spraints on the River Ticino and spectra similarity with Tic-49 sample (%T = trasmittance; cm^{-1} = Frequency).

3. Quantitative relationships for the identification of *S. glanis* in otter's diet

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Quantitative relationships for the identification of *S. glanis* in otter's diet

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ABSTRACT

Bones of *S. glanis* were found in otter's stools from Ticino River (Northern Italy), proving the presence of this allochthonous species in otter's diet. In order to develop specific morphometric relationships between fish mass and undigested remains for this species, we analyzed bones from 28 individuals of *S. glanis* of different size, sampled in the same river. Caudal vertebrae and two basicranial elements emerged as significant elements useful for identification which were conserved in stools. Basicranial elements were present in pairs of the same size and, thus, their dimension was directly related to the fish mass. For vertebrae, we proposed an index, called K index, able to identify the position of a vertebra along the vertebral column. This index increased from 0.7 to 1.4 along the column in the cephalic-caudal direction and it was independent from the fish size. Along the vertebral column, five 'K' groups were defined for practical porpoises and five different equations were proposed. This methodology allowed to established the size of the preyed fish, opening new perspectives for more detailed studies on the efficiency of predation on this allochthonous species.

Keywords: otter diet; *Lutra lutra*; bone remains; *Silurus glanis*; morphometric relationship

INTRODUCTION

The European catfish (*Silurus glanis*), also known as wels catfish or sheatfish, is a large predator native to Eastern Europe and Western Asia (Stone 2007). It has been introduced into many European basins in France, Italy, the Netherlands, Spain and UK mainly for sport fishing and aquaculture purposes (Keith & Allardi 2001; Copp et al. 2009). The large size and relatively frequent capture of *Silurus glanis* increase its popularity among fisherman leading to irregular introductions in different countries (Copp et al. 2005). Nowadays, the ecological effect of this species is still debated. In waters in which fish species have evolved in the presence of native piscivorous fish (e.g. *Esox lucius*), the potential impact of *S. glanis* was considered as shallow (Hickley and Chare 2004), while in habitat with only a few or no native predatory species, the introduction of *S. glanis* has been reported to have conflicting impact on native communities (Copp et al. 2009). In fact, some authors reported that *S. glanis* wasn't a voracious predator, but an opportunistic scavenger (Copp et al. 2009), and a poor biomanipulator (Wysujack & Mehner 2005). On the contrary, Zerunian et al. (2002) considered the presence of *S. glanis* as threatening because of the high reduction in number of indigenous species consequent to its introduction. In Italy, *S. glanis* has been introduced in the early 20th century: the first record was in 1937 and was related to a specimen caught in the Adda River (Po basin, Northern Italy) (Manfredi, 1957). By the 1980s this species was considered as common (Gandolfi & Giannini, 1979).

The river otter (*Lutra lutra*) is an aquatic top predator, which represents a key and umbrella species for inland aquatic habitats (Ruiz-Olmo and Jimenez, 2009; Clavero et al., 2010; Almeida et al., 2012), contributing to sustain the ecological balance and the biotic integrity of riverine ecosystems (Prenda et al. 2006; Miranda et al., 2008). Fish are the staple of otters diet, constituting up to 95% of their preys (Ruiz-Olmo and Palazon, 1997), which however can feed also on amphibians, crayfish, and reptiles (Clavero et al., 2003). Introduced and native fish species can be equally preyed by otters (Lanszki & Sallai 2006; Brzeziński et al 2006; Miranda et al 2008; Gorgadze 2013).

During an otter monitoring campaign on the Ticino River (Northern Italy), many undigested parts of *S. glanis* have been observed in otter stools. This finding was new for this area (Smiroldo et al., 2009), and well agreed with the recent fish monitoring on this river, which revealed an increasing presence of alloctonous species, and a particularly high density of *S. glanis*.

The aim of this paper was to identify, when possible, the presence of *S. glanis* bones in otter feces surveyed on Ticino River. Moreover, by using bones from fish of different size sampled in the same river, we performed a morphometric relationship study between fish mass and bone size in order to quantify the role of *S. glanis* in otter diet.

MATERIAL AND METHOD

Otter survey and diet analysis

Otter's stools, were collected from March 2016 to March 2017 in the Ticino River (NW, Italy), one of the main tributaries of the Po River (Fig. S1 in Supplementary Information), with an overall length of 284 km. The river stretch that flows downstream Lake Maggiore is preserved by two Regional Parks covering 918 and 66 km^2 of Lombardy and Piedmont, respectively. This portion flows in a plain area characterized by intensive agriculture and anthropogenic settlements (Garzoli et al. 2014). Nevertheless, woodlands and natural riverine habitats still cover most of the area. Large riparian woods are composed of english oak (*Quercus robur*), european white elm (*Ulmus laevis*) and field elm (*Ulmus minor*), european ashes (*Fraxinus excelsior/Fraxinus angustifolia*), and alluvial forest composed by alder (*Alnus glutinosa*), willows (*Salix alba* and *S. eleagnos*), and poplars (*Populus alba* and *P. nigra*). Based on biological, hydromorphological and chemical parameters the ecological condition of the river is good (EU, 2000), even if in its Southern part the biological parameters and the concentration of hexachlorocyclohexanes (HCHs) exceed the current limit values according to 2000/60/EU Water Framework Directive (ARPA, 2014).

Otter monitoring was performed following the 'Standard Method' recommended by the IUCN/SSC Otter Specialist Group (Reuther et al. 2000). 32 sampling stations (Fig. S1) along the whole sublacual portion of the Ticino River have been considered (average distance among stations was 5 km and mean length ± SD of each station was 567±263 m). detail of the sampling stations and the monitoring results are reported in Smiroldo et al, 2019). Briefly, each otter's feces was wrapped in aluminium foil, put in plastic bag, labelled, and transported to the laboratory where each sample was soaked for 24 hours in a solution of distilled water and hydrogen peroxide. All samples were then washed through a sieve of 0.5 mm mesh, dried in oven and observed under a stereomicroscope (Wild M3Z 6.5-40x, UK). Fish remains were identified from their vertebrae, jawbones and scales using personal collections and keys from different authors (Webb 1976; Wise 1980; Camby et al. 1984; Prigioni 1997).

Laboratory collection of S. glanis bones

28 European catfish were taken from the lower part of the Ticino River in summer 2017 by selective electrofishing, during a *S. glanis* containment program. In laboratory, each individual was labeled with an identification code and measured. According to Rutkayova (2013), total and eviscerated weights and 12 morphometric determinations were recorded for each individual (Tab. S1 in Supplementary Information). Fish were manually cleaned by removing their visceral parts, wrapped in tinfoil and cooked in the oven for 2-3 hours at 150°C. According to Rojo (2013), the organic parts bound to the skeleton were removed with larvae of *Hermetia* sp. (Diptera, Stratiomyidae). Clean bones from each individual were taken separately and used for skeleton reconstruction and morphometric measurements. *S. glanis* bones and those coming from otter's spraints were observed and photographed by a Leica stereomicroscope with an integrated digital camera (Leica EZ4D). Measurements have been performed on images using the ImageJ free software (Schneider et al. 2012).

RESULTS AND DISCUSSION

One of the most important aspect of animal ecology and ethology is the study of the diet. Nowadays feces analysis is the most common method for assessing diet, especially through undigested bones recognition, even if in the otter case several problems have been reported (Putman 1984). In diet analysis, fish remains are generally identified from their vertebrae, jawbones and scales, using personal collections and/or keys from different authors (Webb 1976; Wise 1980; Camby et al. 1984; Prigioni 1997). Today, due to the presence of alien species, these keys need to be implemented (Savini et al. 2010). One of the most useful tools to recognize fish remains in piscivores species are otoliths; these undigested remains composed of calcium carbonate are exposed to a variable degree of chemical degradation and mechanical abrasion in the digestive track of predators (Pierce and Boyle 1991; Pierce et al. 1993). Furthermore, small otoliths are likely to be totally dissolved and hence some species may fail to be detected (Granadiero & Silva 2000). In the present work we focus on vertebrae; being mineralised (calcium phosphate), these structures are expected to be more resistant than otoliths, and consequently more easily traceable in the feces of piscivore species (Pierce et al. 1993). Moreover, vertebrae has different peculiarity useful for species determination such orientation of dorsal and ventral zygapophysis, size and shape of neural and haemal spines, sculpturing of the centrum and location and size of foramina (Desse & Du Buit,1970, 1971; Casteel, 1976; Watson, 1978; Pierce & Boyle 1991; Pierce et al. 1991a; Watt et al. 1997). Vertebrae were used also for the minimum number of prey and to back-calculate their size (Feltham and Marquiss1989; Feltham 1990; Desse & Desse-Berset 1996a, 1996b). The quantification step is a critical point which must balance precision and practicality. The proportional quantification of bone weights coming from different items in a stool is a practical option able to estimate the relative amount of each food item from the overall amount of bone remains, but is unable to distinguish the pray number and their relative size. On the other hand, the quantification based on mathematical equations, which relate the size of a specific bone to fish weight, is a time-consuming methodology able to assess the weight of preyed individuals. This option was chosen, addressing our attention to *S. glanis* bone remains found in otter's stools coming from Ticino River.

Comparing undigested remains in otter's stools and samples coming from our laboratory collection, the caudal vertebrae and two basicranial elements emerged as significant diagnostic elements, as shown in Fig. 1.

The typical alveolar structure with 'hourglass' shape on the vertebral body appeared as diagnostic element, useful in species identification (Fig. 2). This structure has some similarities with that of trout (*Salmo trutta*), even though substantial differences in the weft of the pores and in the absence of the 'hourglass' structure are present (Fig. 3). Vertebral neural processes are also significantly different, but it must be considered that they represent labile structures during the mastication process and manipulation in laboratory. To support the analyses of *S. glanis* bones, two additional morphological characteristics of the vertebrae have been identified: an anterior-dorsal (AD) and an anterior-ventral process (AV) (Fig. 4). These processes differed along the vertebral column with the lack of the AD process in the first vertebra and a frequent double AV process in the first vertebrae.

The second diagnostic elements found in feces are the basicranial elements (Fig. 5) which are present in pairs and with the same size in each individual. Length and width measurements were carried out on 28 samples from our laboratory collection (Tab. S2) and both measures resulted related to the known fish mass. We thus propose the two equations available in Table 1 to be used for predictive porpoises and when only one dimension could be measured, the value of this element was kept as sufficient for fish mass quantification.

Since bone remains in stools are usually limited in number and difficult to identify for sure, the identification of the exact position of each vertebra along the vertebral column results as a very hard task. For this reason, we selected from our collection 5 samples with a total weight that ranged between 10 and 323 g and from each sample we measured the height and the mean diameter of each vertebra to find and then propose an index that allows an accurate identification of the vertebral position along the column. The height was calculated as the distance between the two transversal planes delimiting the vertebral body (Fig. 6a), while the mean diameter was calculated as the mean value of the two orthogonal diameters passing through the medulla hole in the middle of the vertebra (Fig. 6b). The ratio between these dimensions (height-diameter ratio) increases along the vertebral column in the cephalic-caudal direction between 0.7 and 1.4 for all the considered fish 8in the range between 5 and 323 g, and thus it was independent from the fish size (Fig. 7). The increase of the height-diameter ratio depends by the fact that the mean diameter (denominator) decreases more rapidly than the vertebral height (numerator). Consequently, the index obtained called ‘K’ index, changed greatly along the vertebral column. Considering the numerical range of the K index (0.7-1.4) we have divided it into five biometric groups for practical reasons: up to 0.9; from 0.91 to 1.0; from 1.01 to 1.1; from 1.11 to 1.2; above 1.21. The first and the last group have an open interval higher than the others. The reason of this difference is that at the beginning and at the end of the vertebral column, the k index values varied greatly for a lower number of vertebrae, more than in the central portion of the column. The number of vertebrae falling in each group ranges between 7 and 13 and the mean value of the k index of vertebrae falling in the same group is very similar or identical to the central value of the interval and its variability, calculated as a standard deviation among the K index of vertebrae falling in the same group, does not exceed the limits of the group.

Once identified a method to assign each vertebra to a specific group, we were able to define five quantitative relationships for calculating the fish weight from the vertebral size. The mean diameter has been chosen as ‘vertebral dimensional parameter’ because it shows a greater variability range along the vertebral column than the vertebra height. Therefore, the mean diameter of the vertebrae belonging to each group was related to the fish weight, obtaining five regression equations shown in Fig. 8. In this way, a vertebra found in otter feces can be assigned to a ‘k’ group, by its K index (relative position in the vertebral column), and the weight of a predated individual, as it was before ingestion, can be assessed through the relationship between the mean diameter and the fish mass of that group (Tab. 2). In the case that several vertebrae found in the same stool give the same weight, it is probable that they belong to the same individual, whereas vertebrae, that provide different weights, could belong to different individuals.

In this work, 13 osseous components, coming from 8 different stools, were particularly intact, 12 caudal vertebrae and a pharyngeal-basicranial element (Tab. 3). According to our methodology, diagnostic elements in the same stool were attributed to the same individual, if the resulting fish weight was very similar (e.g. estimated fish weight of 10 and 9 g in sample Tic 58). On the contrary, when estimated fish weight was very similar but the diagnostic elements came from different stools, they were attributed to different individuals (e.g. estimated fish weight of 240 and

241 g in samples Tic 28 and Tic 57, respectively). The third possibility was the finding of diagnostic elements in the same stool giving very different estimated weights (e.g. estimated weight of 5 and 15 g in sample Tic 26). In this case two different individuals were considered. In total we were able to identify10 individuals in 8 stools having a mean weight of 150 ± 179 g with 5-556 g of minimum-maximum interval.

To our knowledge, the presence of *S. glanis* in otter feces has never been identified in Italy. Due to the increasing diffusion of alien species, reaching a deeper knowledge of the interactions among native and non-native species is a crucial point for better address future conservation strategies. Keystone species in particular (Power et al. 1996), such as Eurasian otter, can have an essential role in restraining non-native species by predation, with an important role in the conservation and rehabilitation of ecosystem biotic integrity (Prenda et al. 2006).

The presence of *S. glani*s remains in stools coming from the Ticino River highlights the potential role of this mammal for controlling the populations of this allochthonous species. Alien food resources are expected to have major effects on the populations of food limited predators (Beja, 1996) and may enhance the survival of rare or declining species of conservation interest. Contrasting studies reported the effects of otter predation on autochthonous species: in Southern Italy, non-native fish (*Micropterus salmoides* and *Lepomis gibbosus*) have been reported to be preyed less than expected by otter (Prigioni et al. 2006), while an increase in non-native fish consumption has been observed in Portugal during the flooding of an artificial lake (Pedroso, Sales-Luìs & Santos-Reis, 2011). Similarly, in Loch Lomond (Scotland), an introduced species (*Gymnocephalus cernuus*) has become the main prey of otters, but only 20 years after its introduction (McCafferty 2005).

CONCLUSION

From our analyses we can report for the first time in Italy the predation of the European otter on *S. glanis.* Considering the increasing diffusion of this species in the Italian rivers and according to other authors, we can conclude that otters are opportunistic predators, which feed on the most abundant prey and, thus, following the increase of *S. glanis* in Italian rivers, started to feed on this species too. This makes the European otter an important element for controlling allochthonous fish species confirming its keystone role for the conservation of the ecosystem biotic integrity.

Beside the proven presence of *S. glanis* bones in otter’s feces, our proposed equations and relative K indexes allowed to established the size of the preyed fish from a morphometric analysis, thus opening new perspectives for more detailed studies on otter's diet and on the efficiency of predation by otters.

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Tab. 1 Equations relating the two measurements performed on the basicranial elements

	Equation	
Lenght	$y = 9,8844\ x^2 - 47,405\ x + 60,701$	$R^2 = 0,98909$
width	$y = 0,8066\ x^{3,9544}$	$R^2 = 0,96853$

Table 2 Equations related to the five K groups (x = average diameter of a vertebra, y = estimated weight of the fish)

K Group	Equation	
I	$y = 1,2544\ x^{3,6517}$	$R^2 = 0,99529$
II	$y = 30,359\ x^2 - 69,158\ x + 45,018$	$R^2 = 0,99427$
III	$y = 5,434\ x^{3,2412}$	$R^2 = 0,95767$
IV	$y = 7,8421\ x^{3,6157}$	$R^2 = 0,9814$
V	$y = 18,056\ x^{3,6038}$	$R^2 = 0,98811$

Tab 3 Remains of *S. glanis* data found in faecal samples in favorable conditions for calculating K index and weight. C.V. cervical vertebrae; B basicranial sample.

Stool sample	Date	Bone sample	Average Ø (mm)	Height (mm)	K index	K group	Weight (g)
TIC 25	22/10/2016	C.V	4,31	3,43	0,80	I	260
TIC 26	22/10/2016	C.V	0.99	1.03	1.04	III	5
TIC 26	22/10/2016	C.V	1.98	1.69	0.86	I	15
TIC 27	10/09/2016	C.V	3.25	3.6	1.11	IV	556
TIC 28	10/09/2016	C.V	3.22	3.25	1.01	III	240
TIC 57	28/01/2017	C.V	2.58	2.87	1.11	IV	241
TIC 58	28/01/2017	C.V	0.86	1.19	1.38	V	10
TIC 58	28/01/2017	C.V	0.73	1.13	1.55	V	9
TIC 61	28/01/2017	C.V	2.26	1.7	0.75	I	25
TIC 61	28/01/2017	C.V	1.4	1.44	1.03	III	16
TIC 61	28/01/2017	C.V	1.54	1.58	1.03	III	22
TIC 95	18/03/2017	C.V	1.8	2.2	1.22	V	150
Stool sample	**Date**	**Bone sample**	**Lenght (mm)**		**Weight (g)**		
TIC 58	28/01/2017	B	2.75		5		

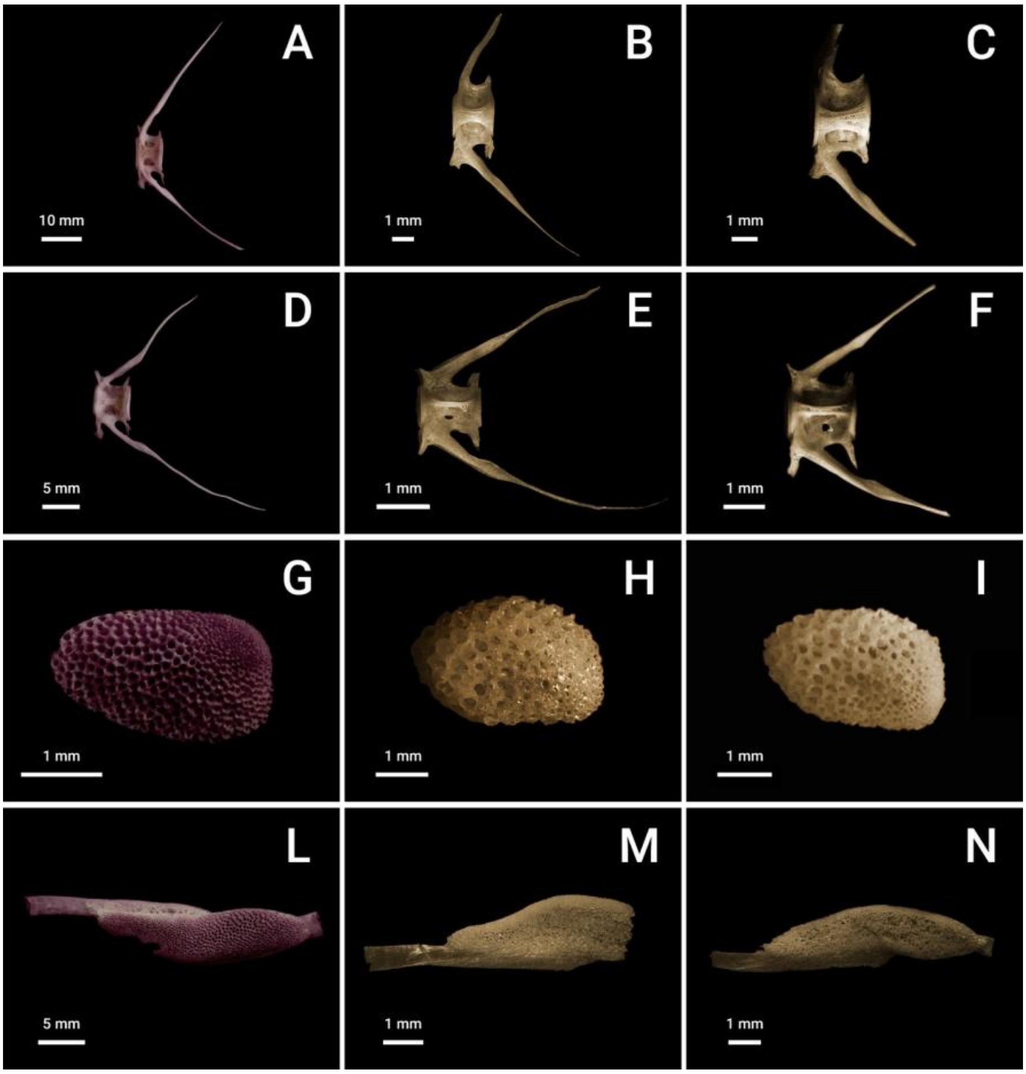

Fig1: photos of the samples from the Archaeological Fish Resource photographic archive of the University of Nottingham (http://fishbone.nottingham.ac.uk/); Centrum: remains from clean fish sample; right: remains from spraint analysis

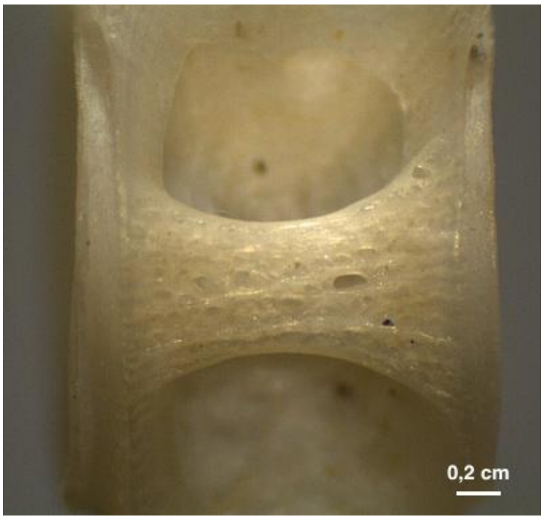

Fig2: Typical alveolar structure with ‘hourglass’ shape on the vertebral body.

Fig3: Vertebrae trout (*Salmo trutta*) with alveolar structure without the 'hourglass' shape on the vertebral body.

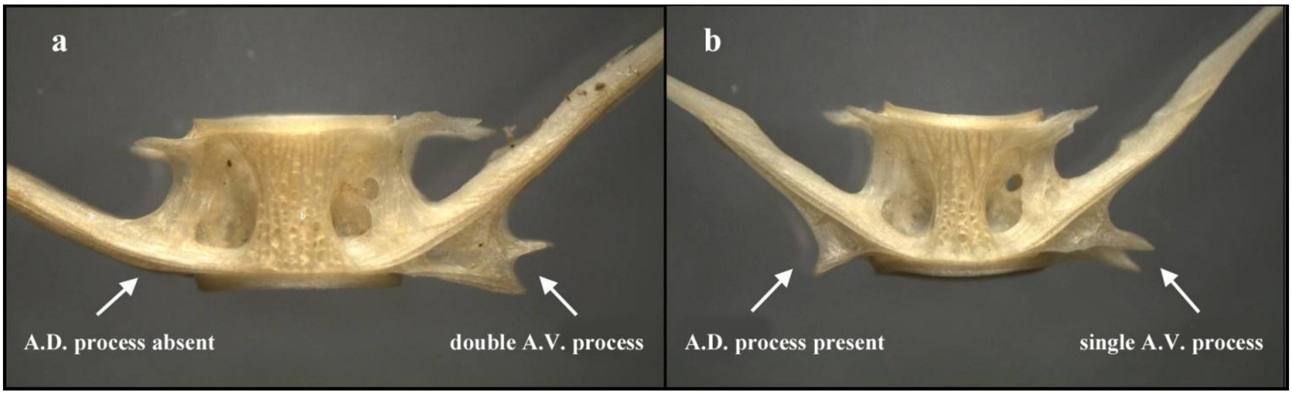

Fig4: Anterior-dorsal (AD) and an anterior-ventral process (AV) – A: with the lack of the AD process in the first vertebra B: double AV process in the first vertebrae.

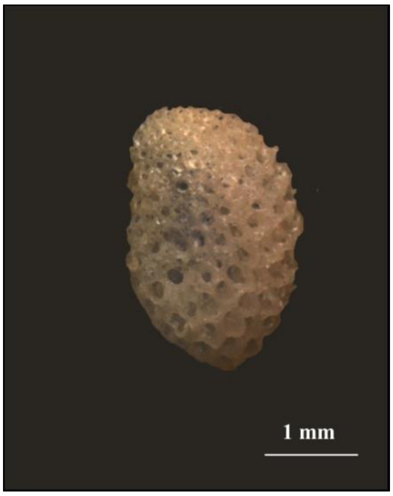

Fig5: Basicranial elements.

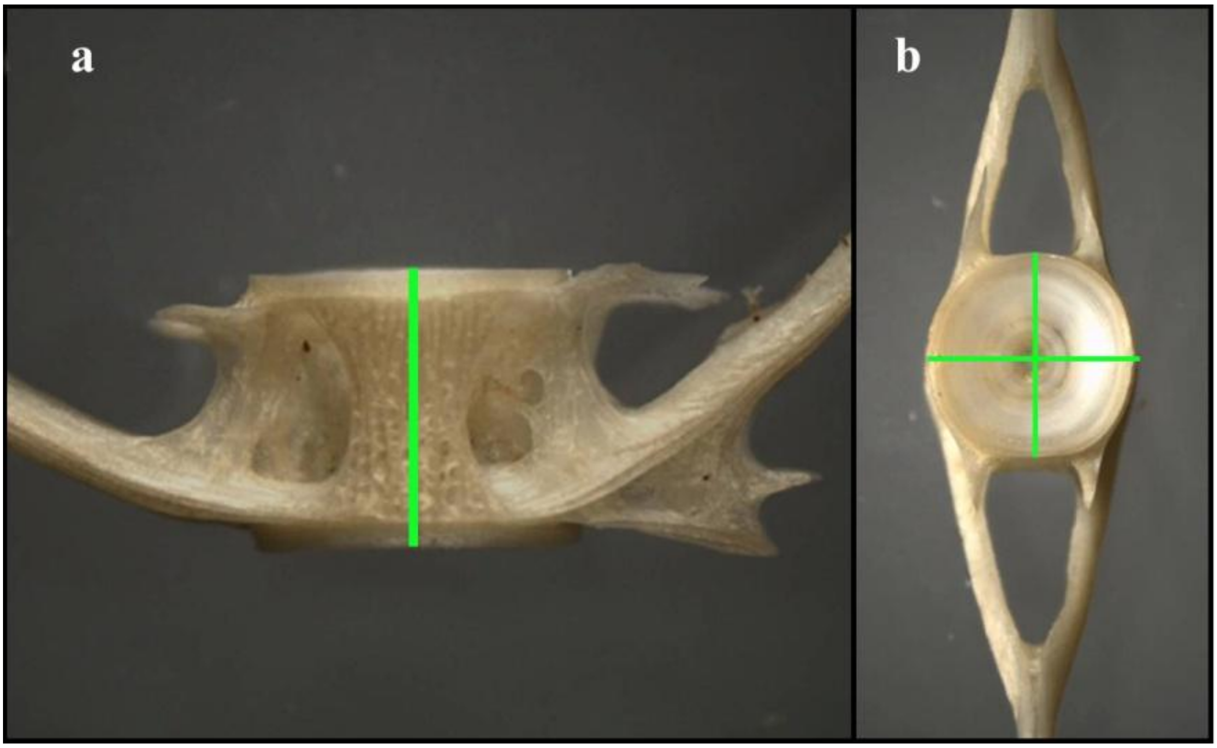

Fig 6: (a) The height as the distance between the two transversal planes delimiting the vertebral body; (b) mean diameter calculated as the mean value of the two orthogonal diameters passing through the medulla hole in the middle of the vertebra.

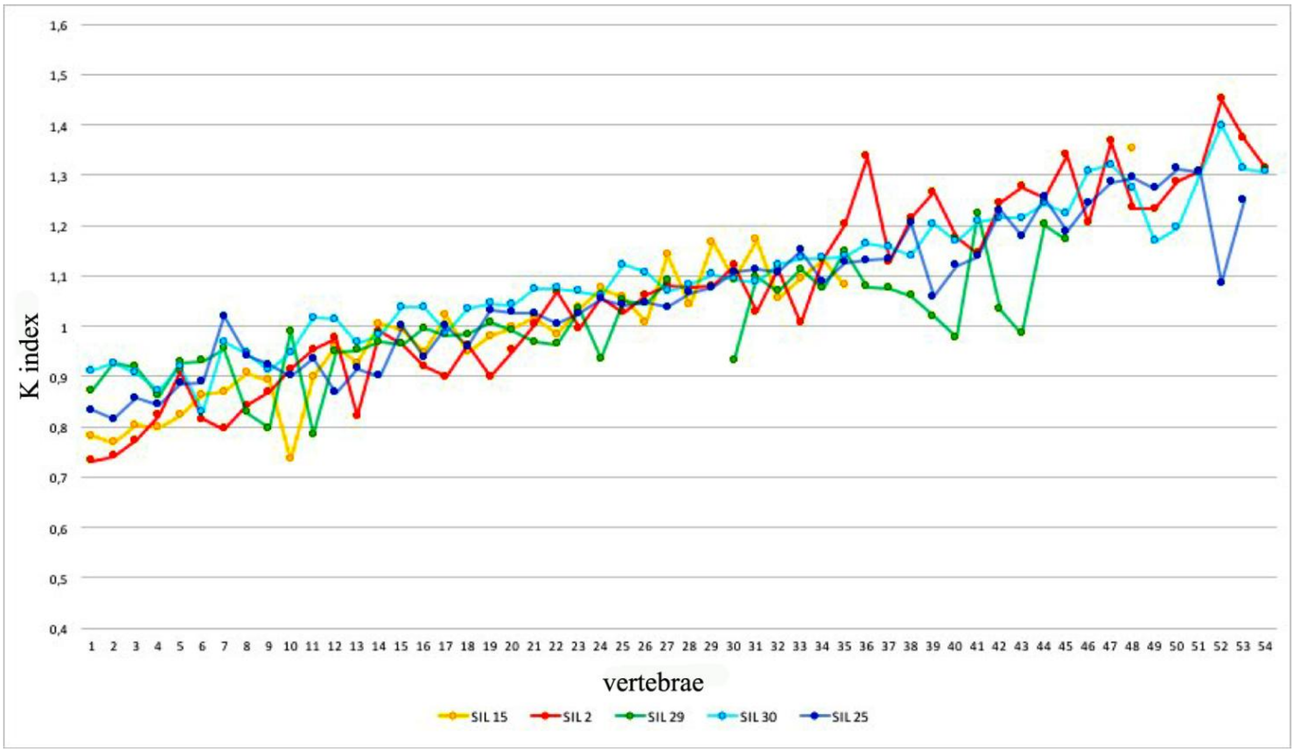

Fig7: The increasing ratio between height and diameter along the vertebral column in the cephalic-caudal direction all the considered fish sample (Sil 15; 2; 29; 30; 25).

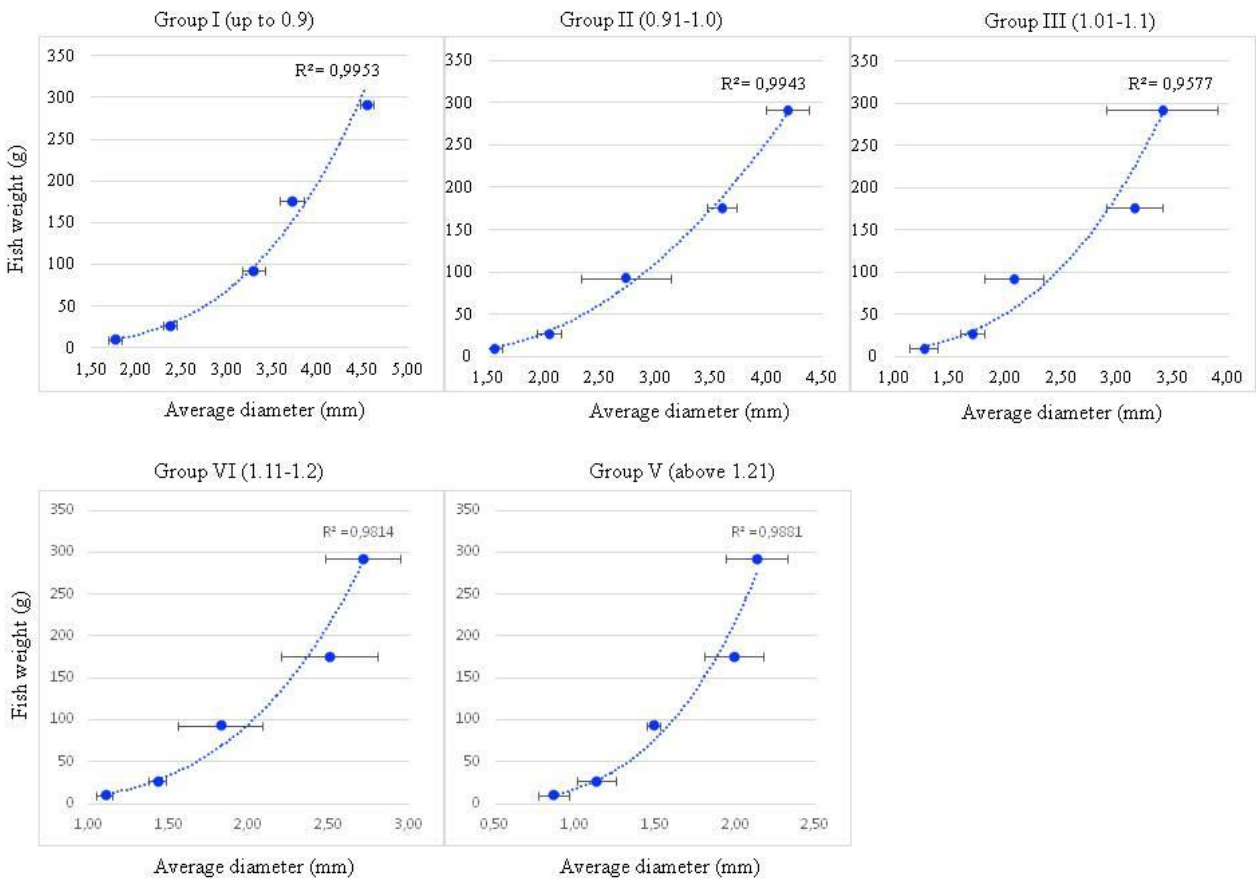

Fig8 : relation from the mean diameter of the vertebrae belonging to each group was to the fish weight

FigS1: Attach: Morphometric data relative to the 28 samples of *S. glanis* according to Rutkayova 2013. n°= number of *S. glanis* sample; tl = total lenght (cm); sl = standard lenght (cm); tw = total weight (g); vw = visceral weight (g); ew = eviscerate weight (g); lprp = distance praepectoralis (prepectoral length) (cm); ol = opercular lenght(cm); pfl = lenght pectoral fin ray (cm); dv-a = distance ventral-anal (cm); vfl = ventral fin length (cm); dfl = dorsal fin length (cm); afl = anal fin length (cm); de = diameter eye (cm)

n°	**tw**	**ew**	**gw**	**tl**	**sl**	**lprp**	**ol**	**pfl**	**dv-a**	**vfl**	**dfl**	**afl**	**de**
2	27	25	1	19	17.6	3.2	3.7	2.4	1	1.4	1.1	10	0.3
3	19	15	3	16.8	15	3	3.4	2	0.8	1.2	1	8.2	0.25
4	34	31	2	19.5	18.9	3.4	4	2.2	1	1.7	1.4	11.1	0.3
5	23	21	1	17.8	16.1	3.2	3.5	2.2	0.5	1.3	1.3	9.3	0.25
6	35	31	4	20.2	19	3.6	3.8	2.5	1	1.7	1.1	10.9	0.3
7	25	23	2	17.7	15.9	3.2	3.4	2.3	0.5	1.3	1.1	9.2	0.25
8	88	80	7	27.5	24.5	4.6	5	3.1	0.6	2	1.8	14.5	0.4
9	25	23	1	18.2	16.1	3.1	3.2	2	0.6	1.4	1.2	9	0.25
10	36	31	2	20	17.6	3.8	4	2.6	0.6	1.6	1.3	10	0.25
11	58	52	4	23.5	21.2	4.1	4.4	3	0.5	1.8	1.5	12	0.3
12	60	53	6	24	21.3	4.3	4.7	3	1	1.8	1.6	13	0.3
13	16	15	1	16.3	14.6	3	3.3	1.8	0.5	1.2	1.1	8.7	0.25
15	10	9	1	14.2	12.4	2.6	2.8	1.9	0.5	1	1.1	7	0.2
16	22	20	1	17	15	3.1	3.3	2.2	0.8	1.3	1.2	9.2	0.25
17	62	57	4	23.5	21.4	3.8	4.3	2.8	0.8	1.5	1.5	13	0.3
18	47	43	4	21.6	19.4	3.5	3.8	2.5	1	1.5	1.4	12	0.3
19	22	21	1	17.7	16	2.9	3.2	2	0.5	1.2	1.1	9.8	0.25
20	20	19	1	17.2	15.8	2.7	3.2	2	0.7	1.1	1.1	9	0.25
21	135	124	10	29	26.3	4.8	5.5	3.5	0.9	2.2	1.8	15.2	0.35
22	16	14	1	16	14	2.8	3	2.1	0.5	1.1	1	8.5	0.25
23	10	9	1	14	12.5	2.4	2.7	1.6	0.4	1	1	8	0.2
24	261	246	19	36	32.3	6.5	6.5	4.3	0.9	2.7	2.2	20	0.45
25	292	269	19	37.3	33.7	6.7	6.7	4.6	1.4	2.8	2.2	21	0.5
26	176	161	14	32.3	29	5.4	5.8	2.5	1	2.5	2	18	0.45
27	249	231	10	35.5	31.5	6.5	6.8	4	1.3	2.5	2.2	19	0.45
28	323	284	30	38	33.8	7	7	5	1.2	3	2.5	21	0.5
29	93	86	5	24.5	22.5	5	5.3	2.8	0.9	2	1.7	12.3	0.25
30	176	163	12	31.5	28.2	5.5	5.5	3.5	1	2.5	1.8	16	0.4

4. Predation on amphibians may enhance eurasian otter recovery in southern italy

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PREDATION ON AMPHIBIANS MAY ENHANCE EURASIAN OTTER RECOVERY IN SOUTHERN ITALY

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ABSTRACT

Mediterranean freshwaters undergo extreme seasonal variation in water flow, which, exacerbated by water withdrawal for agriculture or hydroelectric purposes, may affect fish communities and thus prey availability for semi-aquatic predators, such as Eurasian otter *Lutra lutra*. To investigate the role played by food availability on the ongoing recovery of an otter population at the southernmost limit of its Italian range, we assessed otter diet by the analysis of 357 spraints collected from 2014 to 2017 on eight rivers, and both fish and amphibian availability by, respectively, electrofishing and visual encounter surveys. Fish and amphibians formed the bulk of otter diet, the latter resource contributing as much as fish to otter diet in spring. Use by otters of both fish and amphibians depended only fish availability, suggesting that amphibians constituted an alternative resource to be exploited in conditions of fish shortage. Accordingly, electrofishing showed that fish biomass may be barely sufficient to sustain the current otter population. ATR-FT-IR spectroscopy allowed to point out for the first time the occurrence of amphibian eggs in otter spraints, although the co-occurrence of anuran bones did not allow to discriminate between direct and passive predation. Overall results indicate that the expansion or even survival of this small otter population may depend on the effective management of water resources and reinforcement of fish assemblages.

Keywords: river otter, predation, Mediterranean diet, opportunistic behavior, spectroscopy, amphibian eggs.

INTRODUCTION

The Eurasian otter (*Lutra lutra*) is a 'sentinel' key species of European inland waters (Ruiz-Olmo and Jimenez, 2009; Clavero et al., 2010, Lemarchand et al., 2011; Almeida et al. 2012), preying essentially on aquatic and semi-aquatic species (Mason and Macdonald, 1986; Kruuk, 1995, 2006) (Chanin, 2003; Miranda et al., 2008). Following the dramatic decline in numbers and range occurred in the second half of the 20th century (Ruiz-Olmo et al., 2008), since the 1990s otter populations have started to recover in many countries (Kranz and Toman, 2000; Conroy and Chanin, 2002), as a consequence of the banning or regulation of Persistent Organic Pollutants (POPs) and hunting restrictions (Ruiz-Olmo et al., 2000).

The trend of the Italian otter population has followed the same pattern (Prigioni et al., 2007). Currently, otters are expanding in central and southern Italy and sites positive for otters have been recorded in the north-eastern part of the peninsula, as a consequence of the expansion of Austrian and Slovenian populations; in addition, a small reintroduced B-line population occurs in Lombardy region (Balestrieri et al., 2016). Despite this positive trend, otters in Italy are still threatened by several factors, including the destruction of river habitats, agriculture and urban intensification, water pollution, poaching, road traffic (Loy et al., 2015). Food shortage and isolation are considered the main current threats to Italian otters (Panzacchi et al., 2010). For these reasons, and because the size of the Italian population is still lower than 300 individuals (Prigioni et al. 2006a), according to IUCN's criteria the otter is listed as "Endangered" (EN).

The semi-aquatic lifestyle of river otters is metabolically costly, requiring a daily consumption rate between 16% and 22% of their body mass (Kruuk, 2006); thus otters are voracious hunters, but with the most restricted trophic niche in the mustelid family (Jedrzejewska et al., 2001; McDonald, 2002). Wherever fish supply is not limiting, the otter is almost exclusively piscivorous (Ruiz-Olmo et al., 1989; Prigioni et al., 1991), whilst it can show a plastic feeding behaviour in the opposite case (Delibes and Adrián, 1987; Beja, 1996; Ruiz-Olmo et al., 2001; Remonti et al., 2008; Ayres and García 2009, 2011; García-Diaz and Ayres 2010; Román, 2011; Almeida et al., 2013). Being influenced by slope, altitude (Johal et al., 2001; Santoul et al. 2004; Ibanez et al., 2007) and anthropogenic factors (hydraulic structures and over-fishing; Nilsson et al., 2005; Perez-Quintero, 2007; Wang et al., 2011), fish species richness and abundance may vary both locally, along the upstream–downstream gradient, and at continental scale, increasing south-eastwards throughout Europe (Balestrieri et al., 2013). In Mediterranean regions, climatic fluctuations, have been reported as the main factor affecting fish availability to otters (Ruiz-Olmo et al., 2001; Clavero et al., 2003; Ruiz-Olmo and Jiménez, 2009); consequent to drought (Clavero et al., 2003), in summer otters are mainly piscivorous on main rivers, but tend to rely on alternative food resources on their tributaries, where water availability can be strongly lowered (Smiroldo et al., 2009).

Secondary prey taxa are, in decreasing order of importance, amphibians, crustaceans, birds and reptiles, while insects and mammals are preyed only occasionally (Mason and Macdonald, 1986; Jedrzejewska et al., 2001). Amphibians and crustaceans (*Potamon fluviatilis* and *Austropotamobius pallipes*) are major secondary food resource both in Spain (Clavero et al., 2004) and southern Italy (Remonti et al., 2008). Poisonous skin glands, such as those of toads (*Bufo* spp.) and newts (*Triturus cristatus*, *Lissotriton helveticus*, *L. vulgaris*), do not seem to prevent predation by otters (Ayres and García 2007, 2011; García -Diaz and Ayres 2010; Parry et al., 2015), in which the skinning technique should be innate (Morales et al., 2016).

The southernmost Italian population occurs on the Sila Massif (Calabria region). In this area the otter was claimed to have become extinct in the 1980s and was “rediscovered” in the first half of the 2000s (Marcelli and Fusillo, 2009). Yearly monitoring in 2014-2017 showed that otter presence is stable only on two major rivers and suggested that the current population may be the result of the recovery of a small one gone undetected during the 1983-85 national survey (Gariano and Balestrieri, 2018). Information on freshwater fish distribution and abundance in southern Italy is still negligible. Available data suggest that fish communities have been deeply depleted by overfishing and altered by introductions (Gallo et al., 2012). Shortage of fish supply may hinder otter expansion or persistence on most depleted rivers, posing at risk the long-term survival of this small population. That said, the assessment of otter trophic niche in relation to food availability at the southern limit of its Italian range is of outermost importance for its management and conservation.

With this aim, we assessed the overall and seasonal otter diets by the analysis of spraints collected in 2014-2017. We predicted that under conditions of fish scarcity otters should rely on secondary food categories, mainly amphibians. To verify this hypothesis we assessed the availability of both fish and amphibians throughout the current otter range on the Sila Massif.

MATERIALS AND METHODS

Study area

This study was conducted on eight rivers flowing from the Sila Piccola massif (southern Apennines, Calabria region, S Italy) to the Tyrrhenian and Ionian seas: Savuto (60 km), Amato (56 km), Corace (48 km), Alli (46 km), Simeri (42 km), Crocchio (38 km), Tacina (65 km), Neto (92 km) and Lese (43 km) (Fig. 1). The study area covered ca. 2040 km^2 from the sea level up to 1928 m a.s.l. (Mount Botte Donato) and was partially included in the Sila National Park (737 km^2). The massif consists of a plateau with gently raising edges and a depressed internal sector, were since the 1920s several artificial lakes have been formed by damming the major rivers. Along the altitudinal gradient, the following vegetation layers occur: "lauretum belt" (0–800 m a.s.l), composed by Mediterranean maquis, stone pine (*Pinus pinea*) and oaks (*Quercus* spp.) mixed forests; "castanetum belt" (800–1200 m a.s.l.), composed by chestnut (*Castanea sativa*), oaks, maples (*Acer* spp.) and alders (*Alnus* spp.); "fagetum belt" (1200–1800 m a.s.l.), composed by *Fagus sylvatica*, *Abies alba*, *Quercus cerris* and *Pinus laricio* (Pavari, 1916). The climate, is typically Mediterranean, with sharp variation in temperature and rainfall depending on the wide elevation range.

Otter monitoring

From 2014 to 2017, 18 river stretches (sampling stations) distributed on eight watercourses were surveyed for otter feces ("spraints") following the 'Standard Method' recommended by the IUCN/SSC Otter Specialist Group (Reuther et al., 2000). In 2014 and 2015, surveys were conducted once a year in July, while in 2016-2017 all stations were surveyed once a season (mid July and November 2016, and early March and late April 2017). The mean length ± SD of sampling stations was 625.4 ± 80.7 m (min-max = 504-851 m). During each survey, shorelines were searched by walking on both riversides and around small islands, looking for typical otter sprainting sites (e.g. large stones, bridges, pool banks, confluences), with reference to Macdonald and Mason (1983) and Prigioni (1997). Spraints were stored individually in silver paper and labelled with an univocal code.

Diet analysis

Each faecal sample was soaked for 24 hours in a solution of distilled water and hydrogen peroxide, then washed through a sieve with 0.5 mm wide mesh, dried in oven and observed under a binocular microscope (Leica Wild M3Z 6.5-40x, UK). Fish remains were identified from their vertebrae, jawbones and scales, using personal collections and the keys of different authors (Webb, 1976; Wise, 1980; Camby et al., 1984; Prigioni, 1997). Amphibians were identified by the keys of Di Palma and Massa (1981), whilst telson, chelae and thoracopods were the main diagnostic features for crustaceans. For each sample, the minimum number of individuals of each kind of prey was estimated by the number and position (left-right) of diagnostic hard parts (as mouth bones for fish, illions for amphibians). When no diagnostic part was found, the remains of a prey were considered to belong to a single individual. The weight of preyed fish was assessed by comparing the size of the jawbones found in otter spraints to a reference collection of jawbones taken from fish of known size. A constant weight was assigned to the other prey items: insects 1 g, amphibians 30 g, reptiles and crustaceans 50 g, birds 100 g. Following Prigioni (1991), results were then expressed in terms of both frequency of occurrence and volume:

1. frequency of occurrence (F%) = (number of spraints containing a specific food items / total number of examined spraints) × 100;
2. relative frequency of occurrence (RF%) = (number of occurrences of an item / total number of occurrences of all items) × 100;
3. percent volume (V%) = (total estimated volume of each food item as ingested / total number of examined spraints) × 100;
4. mean percent volume (mV%) = (total estimated volume of each food item as ingested / number of spraints containing that item) × 100.

The F% of main food items was plotted against their mV% to compare the percentage of feces in which a food occurred with the estimated percentage volume of each food whenever it was eaten. In this plot (x × y)/100 equals the mean per cent volume of each food in the overall diet (mV%), and all points with equal x × y values are connected by isopleths (Kruuk and Parish, 1981).

Trophic niche breadth was estimated by Levins' index $B = 1/(R\ \Sigma p_i^2)$ (Feinsinger et al., 1981), where p_i are the mean volumes of the different food categories and R is the number of food categories (Centrarchidae, Anguillidae, Salmonidae, Cyprinidae, unidentified fish, Amphibians, Reptiles, Crustaceans, Insects and other items; the latter including all items with mV% < 2).

Analysis of unknown remains

Unidentified materials (mainly 0.5–5 mm large, spherical or ovoid remains) were isolated, photographed by a stereo microscope (LEICA EZ4 D with Integrated LED illumination and Digital 3 MP Camera), and stored in glass jars. To exclude laboratory air-borne contamination, five Petri dishes were put on the work bench near the operator during the analysis of faecal samples (McCormick et al., 2014). The chemical composition of unknown materials was assessed by Attenuated-Total-Reflectance and Fourier-Transformed-Infrared Spectroscopy (ATR-FT-IR) (Mintenig et al., 2017), which allow the reproducible and non-destructive collection of infrared spectra from small samples. A Perkin Elmer Universal ATR Sampling Accessory (PerkinElmer, Waltham, MA, USA), consisting of a diamond crystal, was used. After each analysis, the crystal was cleaned with acetone. Spectra were collected using a FT-IR Spectrometer (Spectrum One, PerkinElmer, Waltham, MA, USA), using a spectral resolution of 4 cm^{-1} and 32 scans in the range 4000–650 cm^{-1}, and compared with a spectral customized library, including the spectra of several common plastic polymers (e.g. PET, HDPE, LDPE, PS), natural materials (wool, cotton, cellulose and protein rich materials) and lab products used for purification. Only materials matching reference spectra with a correlation coefficient ≥60% were accepted (Lusher et al., 2013).

Fish and amphibian availability

The availability of main food resources to otters was assessed in April 2017 by sampling the same river stretches surveyed for spraints.

Fish were sampled by electrofishing, which usually provides an effective estimate of fish relative abundance at lower costs than removal sampling (Fièvet et al., 1999; Ross et al., 2001). We used a portable electrofishing apparatus performing two upstream passages, as the second pass always allowed to catch < 25% of the number of fish caught in the first one (Peterson et al., 2004). The length of each section was at least 10 times the river width (mean ± SD=78.2 ± 13.8 m; Verneaux, 1981). For each section, all micro-habitats were surveyed. Percent capturability was calculated for each station and species as: (number of fish captured by the first passage / total number of fish) x 100 (Zitek et al., 2004). Fish were identified to species level, measured, weighed and then released at the site of capture. Throughout the period that fish were retained they were regularly observed for signs of stress due to shortage of oxygen. For each station, total biomass (g) and biomass density (g/m^2) were calculated based on the wetted sampled area (stream length x mean width) and percent capturability. We assumed that fish biomass as assessed in spring represented a reliable index of annual fish availability (Remonti et al., 2008).

Amphibian monitoring was performed using transect visual encounter surveys (VES), the most used survey technique for herpetofauna (Gillespie, 1997). For each sampling station, species were searched for by two surveyors walking on both river banks (Crump and Scott, 1994) and checking all refuges and deposition sites. Individuals were handled wearing latex gloves, to allow both species identification and recording of life stages, and then released at the site of capture. The exact time of both the start of each survey and all observations of adult individuals and egg or tadpole clumps was recorded.

To assess amphibian availability for otters, for each sampling station an index of amphibian abundance was calculated as the sum of all adults and juveniles; wherever only tadpoles or eggs were recorded, the number of egg-clutches was assumed to be a measure of the number of breeding females in the population (i.e. 1 clutch = 1 adult female; Savage, 1961), while numbers of tadpoles were converted in an equivalent number of adult females based on the mean number of eggs per clutch (*Hyla intermedia*: 1000, *Rana italica*: 500, *R. dalmatina* 839, *Bufo bufo*: 5000; Sindaco et al., 2005).

This index probably underestimates the overall abundance of amphibians, but, as for fish, was intended to compare their relative availability to otters at sampling stations.

Statistical analyses

Seasonal and site-related (R. Savuto vs. R. Lese) raw frequency data of food items preyed on by otters were compared by the chi-squared test. Because of repeated tests on related data, the sequential Bonferroni's technique was used to determine the level of significance (Rice, 1989). The relationship between elevation above sea level and mV% of main food items was tested by Pearson's correlation coefficient (r). To test for the hypothesis that otters rely on secondary food resources when fish availability is low, the relation between the mV% of both fish and amphibians in the monitored stations and either fish biomass density or amphibian abundance was tested by linear regressions. For all analyses, only sampling stations with >10 spraints were used, and all variables were checked for normality by Kolmogorov-Smirnov test ($z < 0.97$ and $P > 0.31$ for all tests). Mann-Whitney's test was used to compare the mean index of amphibian abundance between sampling stations positive- and negative for otters.

RESULTS

Overall, 357 spraints were collected, of which 266 in 2016-2017 (summer: 34; autumn: 30; winter: 85; spring: 117). Otters occurred on 6 rivers (Savuto, Neto, Lese, Tacina, Simeri and Amato) for a total of 12 sampling stations.

Fish formed the bulk of otter diet (F% = 84.3 and mV% = 63.8; Tab. 1, Fig. 2). Cyprinids (chub *Squalius cephalus*, bleak *Alburnus alborella* and roach *Rutilus rubilio*) were the most important fish family (F% = 51.8, mV% = 28.4), followed by salmonids (*Salmo trutta,* Atlantic strain; F% = 36.1, mV% = 18.5) and eel *Anguilla anguilla* (F% = 19.0, mV% = 7.2; Tab. 1). Amphibians were the main alternative-to-fish food category (F% = 46.2, mV% = 31.1), while reptiles and crustaceans (mainly the river crab *Potamon fluviatilis*) were food resources of minor importance (mV% < 5). Insects, birds and fruit were eaten only occasionally.

Fish and amphibians contributed almost equally to otter diet in spring, while predation on amphibians was the lowest in autumn (F% = 4.34 ;χ^2 = 19.1; d.f. = 3; P < 0.01), when cyprinid remains were particularly abundant in otter spraints (F% = 79.3; χ^2 =25.8; d.f. = 3; P < 0.001) (Fig. 3). The percent mVs of fish and amphibians were inversely correlated (r = -0.96, P < 0.001). Reptiles (F% = 11.3; χ^2 =15.7; d.f. = 3; P < 0.01) and salmonids (F% = 61.3; χ^2 =58.8; d.f. = 3; P < 0.001) contributed to otter diet mainly in summer, while crustaceans were more preyed on in winter (F% = 6.9; χ^2 =13.4; d.f. = 3; P < 0.05). Yearly trophic niche breadth (B) was 0.44, ranging between 0.18 in autumn, when otter preyed almost exclusively on fish, and 0.46 in winter.

A significant positive correlation was observed between the mV% of salmonids and elevation (r = 0.71; P < 0.01), while otter predation on cyprinids and eel decreased (r = -0,68; P = 0.01 and r = -0,62; P = 0.03, respectively). Comparing the two major rivers, otters relied mainly on cyprinids (F% = 77.9; χ^2 =64.4; d.f. = 1; P < 0.001) and eel (F% = 27.3; χ^2 =12.3; d.f. = 1; P < 0.001) on the River Lese, while on the River Savuto the frequency of occurrence of amphibians was the highest (F% = 57.8; χ^2 =12.0; d.f. = 1; P < 0.001) and fish and amphibians contributed equally to the overall otter diet (mV% = 53.3 and 44.9, respectively).

Unidentified remains were found in 57 spraints. In 50 samples we found between 5 and 10 spherical or ovoid particles, 500-3500 μm in diameter, with iridescent or translucent amber colorations. ATR/FTIR spectra of 61% of these remains showed peaks typical of protein rich materials and high correlations (63%–96%) were obtained with the spectra of amphibian eggs (see Fig. 4 for an example). Spectroscopy analyses were performed also on fish and crustacean eggs, but their spectra showed lower correlations (< 50%). The identification of unidentified remains as amphibian eggs was supported by the occurrence of amphibians bones in 71% of the spraints containing them. Only one faecal sample contained crustaceans remains but, nonetheless, unknown remains showed a low correlation with crustacean eggs. Moreover, egg-like materials were 97% found in spraints collected in March-April, coinciding with their spawning period in the Mediterranean area.

One filament was identified as fishing wire (79% correlation with Nylon), while 35% of all materials did not match with any reference spectrum (correlations < 65%).

Electrofishing revealed the occurrence of 7 species (Tab. 2). Trout was the only fish species at middle- and high-altitude (> 400 m a.s.l.), on the rivers Simeri, Tacina, Alli, Amato, Savuto and Corace, while cyprinids, mainly chub and roach, predominated below 200 m a.s.l. Fish biomass density differed widely among the monitored stations, ranging from 0.46 to 5.89 g/m^2 (mean ± SD = 3.4 ± 1.4 g/m^2; Tab. 2). Six amphibian species were recorded, of which *Rana italica* was by far the most widespread (Tab. 3). The average index of amphibian abundance did not differ between sampling stations positive- and negative for otter signs of presence (9.0 and 7.4 respectively; $U = 31.0$; $P = 0.92$).

Fish consumption by otters was related to fish biomass ($y = 6.6x+40$; $R^2 = 0.54$; $P = 0.037$), while the use of amphibians showed an opposite trend ($y = -8.0x+57$; $R^2 = 0.56$; $P = 0.033$; Fig. 5).

No significant relationship was found between the mV% of either fish ($y = -0.39x+60$; $R^2 = 0.06$; $P = 0.59$) or amphibians ($y = -0.2x+30.1$; $R^2 = 0.012$; $P = 0.79$) and the index of amphibian abundance.

DISCUSSION

Consistently with previous studies carried out in the Mediterranean biogeographical region (e.g. Ruiz-Olmo et al., 1989; Prigioni et al., 1991; Clavero et al., 2005; Remonti et al., 2008, 2009; Smiroldo et al., 2009), on the Sila Massif fish were the staple prey of otters, with amphibians as the main alternative-to-fish food resource.

Cyprinids, mainly chub, and trout were the main fish prey, and their occurrence in otter diet fatefully reflected their distribution in the study area and, locally, the fish community composition in relation to the upstream-downstream gradient, confirming the opportunistic behavior and feeding plasticity of otters (Smiroldo et al., 2009; Almeida et al., 2012; Balestrieri et al., 2013). Chub, being characterized by a great ecological adaptability (Pompei et al., 2011), is one of the most common freshwater fish in Italy (Gandolfi et al., 1991; Zerunian, 2002) and its gregariousness may facilitate its detection by otters. Trout was the main fish prey in summer, when, compelled by drought to aggregate in small ponds, this fast swimmer may be more vulnerable to otter predation (Magalhães et al., 2002; Prigioni et al., 2006b).

Otters fed on amphibians mainly in spring, when large numbers of amphibians aggregate to spawn, and in winter, during hibernation (Duellman and Trueb, 1986; Weber, 1990; Clavero et al., 2005; Sales-Luís et al., 2007). In spring, ATR-FT-IR spectroscopy allowed to point out the occurrence of amphibian eggs in otter feces. While, to our knowledge, the consumption of this resource by otters has never been reported, mink *Mustela vison* may directly predate on spawns (Day and Linn, 1972; Tolonen, 1982), as so as some monkeys (*Cebus paella*, Neckel-Oliveira and Wachlevski, 2004; *Cercocebus torquatus atys* and *C. diana diana*, Rödel et al., 2002). The finding of egg remains together with amphibian bones did not allow to discriminate if otter preyed either on clutches or on females before spawning. Anyway, predation on amphibian eggs is considered uncommon because their mucoid capsule often makes eggs noxious (Duellman and Trueb, 1986), unpalatable (Kats et al. 1988; Denton and Beebee, 1991), or toxic (Mosher et al., 1964; Light, 1969; Pavelka et al., 1977), although toxic effects may decrease with embryonic development in bufonids (Phisalix, 1922).

As amphibians were exploited by otters during seasons when their availability is the highest and also fish may be more difficult to be preyed upon because of high water discharge and turbidity (Prigioni et al., 2006b), it is hard to discriminate whether seasonal diet shifts depended on fish shortage or amphibian profitability (see also Kruuk, 2006).

In the Iberian peninsula, Ayres and García (2007, 2011) recorded a high common toad (*Bufo bufo*) consumption by otters during their massive breeding period and concluded that toad occurrence depended on their exceptional availability rather than on fish shortage. In our study area the contribution of amphibians to otter diet varied locally, but, on average, the frequency of occurrence of amphibian remains in otter spraints was not related to their availability, as assessed by VES.

Both biomass and species composition of local freshwater communities can vary over time, mainly as a consequence of natural or human-induced variation in discharge (Pires et al., 1999; Scherer and Tracey, 2011; Merciai et al., 2018; Canova and Balestrieri, 2018). If we assume, as for previous studies (Remonti et al., 2008, 2010; Kortan et al., 2010) that our snapshot on the fish and amphibian communities of the study area was representative of their overall relative availability to otters, the relationships between use by- and availability to- otters of these two resources indicated that, on a yearly basis, predation on amphibians would depend on fish shortage. This result is consistent with those of several recent studies (Remonti et al., 2009, 2010; Novais et al. 2010; Karamanlidis et al., 2013; *Krawczyk et al., 2016)* suggesting that, in Mediterranean habitats, amphibians are the otter's major alternative food resource whenever fish availability is limited. Notwithstanding, this does not necessarily prevent otters from focusing on amphibians whenever their disproportionate abundance (i.e. during spawning; Ayres and García, 2011) makes their profitability higher than that of available fish, despite the latter generally provide more energy per gram ingested (e.g. cyprinids: 4.8 kj/g; frogs: 2.9 kj/g; Kruuk, 2006).

Fishing is costly from an energetic point of view and otters have to consume relatively large amounts of food to match energy expenditure (0.8-1.8 kg per day; Kruuk, 2006). As a touchstone, two *adult otters have been reported to consume 8.6–10.8 g/m^2 per year in a stream where salmonid biomass was 9.2–14.4 g/m^2 (Kruuk et al., 1993).* These fish biomass values are similar to those reported for the core area of the Italian otter range (12.7 g/m^2; Remonti et al., 2009), while in our study area mean fish biomass was about one third, such as in streams of the Bieszczady Mountains (SE Poland), for which an unusually intense predation on amphibians by otters has been reported (Pagacz and Witczuk, 2010). Accordingly, on the River Savuto, where otter occurrence has been stable since 2003 (Gariano and Balestrieri, 2018), amphibians played a major role in otter diet, suggesting that the exploitation of this resource may allow otters to cope with fish availability next to the minimum biomass threshold.

Freshwater ecosystems are both the most diverse and threatened biological communities on earth (Allan and Flecker, 1993; Revenga and Mock, 2000; Malmqvist and Rundle, 2002), with the freshwater fish fauna of the Mediterranean basin being particularly at risk because of its high level of endemism (Myers et al., 2000; Olson and Dinerstein, 2002; Abell et al., 2008). Large-scale environmental changes are affecting the availability of several fish species relevant to otters, such as eels, Atlantic salmon *Salmo salar* and brown trout (reviewed by Kruuk, 2006) and ongoing climate change, with increasing temperature and reduced precipitation (Blinda et al., 2007), is expected to exacerbate both water loss and water-allocation conflicts in the next future (Thomas, 2008).

On the Sila Massif, fish community are mainly threatened by anthropogenic pressures, particularly water withdrawal for agricultural or hydroelectric purposes, damming and overfishing (Lucadamo et al., 2012; Gallo et al., 2012). As an example, sampling stations T1 and S4 dried up almost completely in summer 2016-17 and drought, affecting both fish and amphibian availability, may have caused the disappearance of otters from the River Tacina (Gariano and Balestrieri, 2018). Fish diversity is very low, with many sampling stations hosting only one species if not fishless or deeply altered by introductions (Gallo et al., 2012). Under such conditions a relatively small reduction in fish availability may hinder otter expansion or, at worst, reverse its population trend. To prevent the extinction of this almost isolated otter population, conservation management should focus on the regulation of water withdrawal and reinforcing of fish communities.

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COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

GS performed field sampling, conducted diet analyses, discussed, interpreted analysis wrote and edited the paper. PG performed field sampling. AB performed field sampling, wrote and edited the paper. RM performed amphibians sampling. EP conducted chemical analysis and edited the paper. PT wrote and edited the paper.

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Table 1 - Overall diet of otter population as assessed by the analysis of 357 spraints collected in 2014-2017 on the Sila Piccola Massif (South Italy). Data are expressed in terms of both frequency of occurrence (F%, RF%) and volume (V% and mV%; see methods) .

Food items	F%	RF%	V%	mV%
Insects	5.04	2.4	12.6	0.6
Unidentified insect	1.68	0.8	10.3	0.2
Trichoptera	1.68	0.8	6.0	0.1
Coleoptera	0.84	0.4	14.7	0.1
Odonata	0.28	0.1	5.0	0.01
Rhynchota	0.28	0.1	50.0	0.1
Plecoptera	0.3	0.1	30.0	0.1
Arachnida	0.56	0.3	52.5	0.3
Crustaceans	2.52	1.2	52.1	1.3
Potamon fluviatilis fluviatile	2.52	1.2	52.1	1.3
Bivalvia	1.12	0.5	4.0	0.04
Fish	84.3	39.3	75.7	63.8
Centrarchidae				
Perca fluviatilis	1.4	0.7	25.0	0.4
Unidentified Centrarchidae	0.3	0.1	5.0	0.01
Anguillidae				
Anguilla anguilla	19	8.9	37.6	7.2
Salmonidae				
Salmo trutta	36.1	16.9	51.1	18.5
Cyprinidae	51.8	24.2	54.8	28.4
Undetermined cyprinids	10.4	4.8	41.4	4.3
Rutilus rubilio	2.2	1.0	26.3	0.6
Squalius cephalus	34.5	16.1	46.7	16.1
Alburnus alborella	31.7	14.8	23.5	7.4
Undetermined fish	18.2	8.5	51.5	9.4
Amphibians	46.2	21.6	67.3	31.1
Reptiles	3.6	1.7	69.8	2.5
Birds	0.3	0.1	20.0	0.1

Table 2 - Number, length, weight, total biomass (B), biomass density and percent biomass of the fish species found in the 16 sampling stations identified on the 8 monitored rivers.

River	Sampling station	altitude (m a.s.l.)	species	fish number	lenght (cm) min-max	weight (g) min-max	total B g	B density g/m2	B %
Savuto	Parenti	720	*Salmo trutta*	16	14.5-21.4	35-86	1000	3.93	100.0
	Balzata	560	*Salmo trutta*	2	23-26	157-178	335	0.46	100.0
	Attilia	168	*Squalius cephalus*	47	8.5-20.3	9-114	1202	2.40	53.5
			Rutilus rubilio	18	6.5-16.3	7.0-59	653	1.31	29.1
			juveniles	72	4-9	3-10	297	0.59	13.2
			Cobitis taenia	12	7.0-10.5	4.0-9.0	72.5	0.15	3.2
			Carassius carassius	1	10.5	21	21	0.04	0.9
	San Mango	85	*Squalius cephalus*	11	8.0-18.2	8.0-77	378	0.76	31.6
			Rutilus rubilio	8	10.5-17.5	15-84	405	0.81	33.8
			juveniles	51	3.0-8.0	2.0-9.0	193	0.39	16.1
			Cobitis taenia	27	4.0-10.5	3.0-11	136.5	0.27	11.4
			Carassius carassius	1	16	85	85	0.17	7.1
Simeri	upstream	1197	*Salmo trutta*	47	8.2-30	7.0-317	1488	4.13	100.0
	downstream	430	*Salmo trutta*	16	12.8-19.3	24-98	722	2.31	100.0
Tacina	upstream	1355	*Salmo trutta*	13	8.0-27	11-223	820	4.56	100.0
	downstream	764	*Salmo trutta*	12	10.5-37	15-450	825	1.55	100.0
Alli		480	*Salmo trutta*	33	9.0-24.8	9.0-142	1017	2.28	100.0
Lese	upstream	180	*Squalius cephalus*	10	12-18.5	20-68	744	0.71	15.1
			Rutilus rubilio	32	9-16.8	10-58.0	2016	1.92	40.9
			juveniles	227	3.0-9.0	3.0-10.0	2168	2.06	44.0
	downstream	140	*Squalius cephalus*	4	8.3-13.7	7.0-35	160	0.13	6.0
			Rutilus rubilio	11	9.0-16	8.0-69	680	0.57	25.4
			juveniles	108	4.0-8.5	3.0-9.0	1249	1.04	46.7
			Cobitis taenia	3	5.5	3	18	0.015	0.7
			Anguilla anguilla	3	25-50	72-127	568	0.47	21.2
Neto	upstream	173	*Squalius cephalus*	31	4-15.4	3.0-44	589	0.95	16.1
			Rutilus rubilio	59	3.0-18	2.0-83	2413	3.87	65.8
			Anguilla anguilla	5	18-60	26-454	665	1.07	18.1
Corace	upstream	670	*Salmo trutta*	32	8.2-35.8	8.0-543	1855	4.64	100.0
	downstream	520	*Salmo trutta*	30	9.4-27.7	11-238	1972	3.70	98.5
			Rutilus rubilio	1	8.6	8	8	0.02	0.4
			Gambusia affinis	7	4.0-6.0	2.0-4.0	22	0.04	1.1
Amato	upstream	680	*Salmo trutta*	43	8.2-23	9.0-138	1824	3.42	100.0
	downstream	520	*Salmo trutta*	24	10.4-20.8	10-148	1089	3.89	100.0

Table 3 - Results of the amphibian monitoring carried out in the 16 sampling stations. For each station, the relative abundance of amphibians (i.e. their availability to otters) is expressed as an index (I = adults + juveniles + number of clutches + total number of tadpoles/mean number of eggs per clutch; see methods).

River	Station	Species	Adults	Juveniles	Tadpoles	Spawn	I
Savuto	Parenti	*R. italica*	3	-	20	-	9
		P. kl. *berg/hispan*	-	2	-	-	
		B. bufo	-	-	-	4	
	Balzata	*R. italica*	-	-	16	-	1
	Attilia	*R. italica*	-	-	2	-	29
		R. dalmatina	-	-	5	-	
		P. kl. *berg/hispan*	27	-	-	-	
	San Mango	*Pelophyla* spp.	1	-	-	-	2
		B. bufo	-	-	1320	-	
Simeri	upstream	*R. italica*	2	2	-	7	5
		R. dalmatina	-	1	4	3	
	downstrem	*R. italica*	-	1	-	-	13
		R. dalmatina	-	-	-	1	
		B. bufo	-	-	-	11	
Tacina	upstream	*R. italica*	2	1	-	10	29
		R. dalmatina	-	-	-	16	
		B. bufo	-	-	-	10	
	downstream	*R. italica*	2	9	-	-	21
		B. bufo	4	-	-	5	
		S. tergiditata	6	-	-	-	
Alli		*R. italica*	2	3	20	-	5
Lese	upstream	*P.* kl. *berg/hispan*	1	-	-	-	1
	downstrem	*P.* kl. *berg/hispan*	4	-	10	-	12
		H. intermedia	-	-	1	-	
		B. bufo	-	-	1200	6	
Neto	upstream	*R. italica*	1	-	-	-	2
		P. kl. *berg/hispan*	1	-	-	-	
Corace	upstream	*R. italica*	2	2	45	-	23
		B. bufo	-	-	-	19	
	downstrem	*R. italica*	1	-	341	-	39
		R. dalmatina	-	-	30	-	
		H. intermedia	-	-	-	4	
		B.bufo	-	-	-	33	
Amato	upstream	*R. italica*	-	-	100	-	7
		R. dalmatina	-	-	1000	-	
		B. bufo	1	-	-	5	
	downstrem	*R. italica*	2	-	340	-	15
		B. bufo	-	-	-	13	

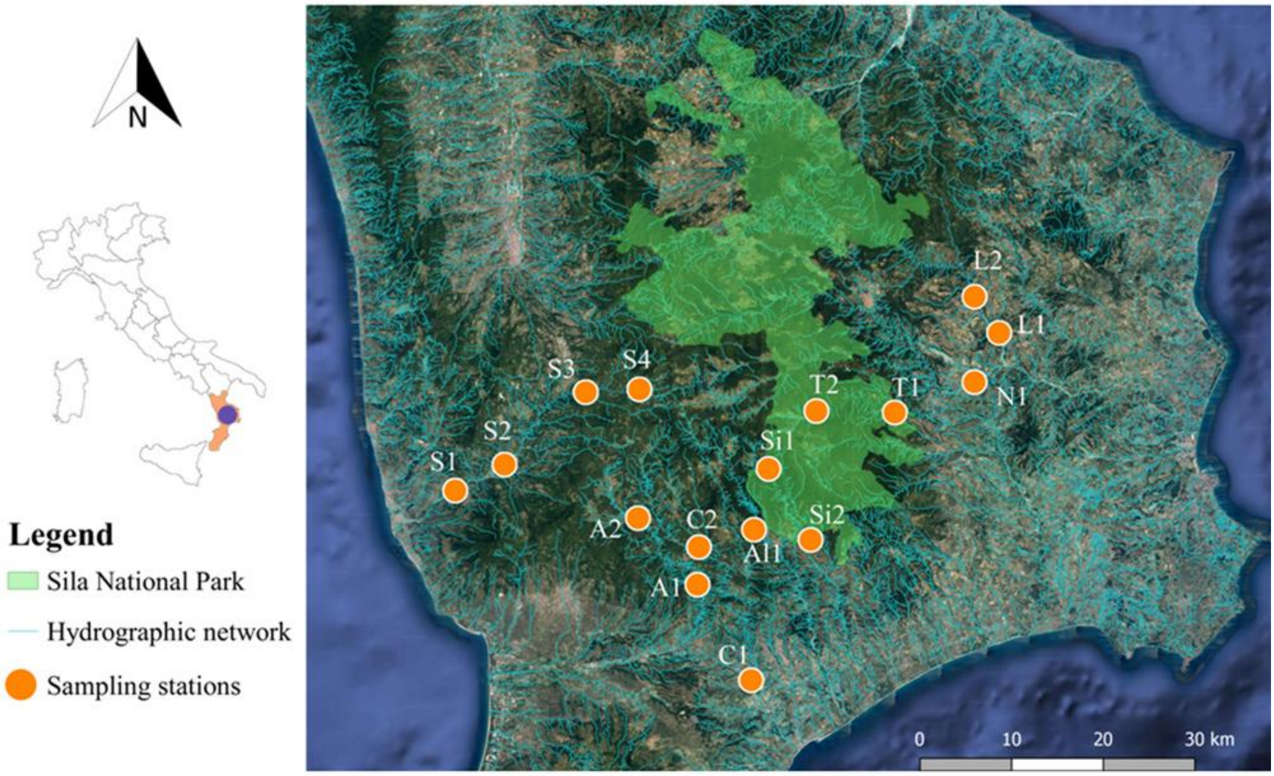

Fig1 Sampling stations on the Sila Massif.

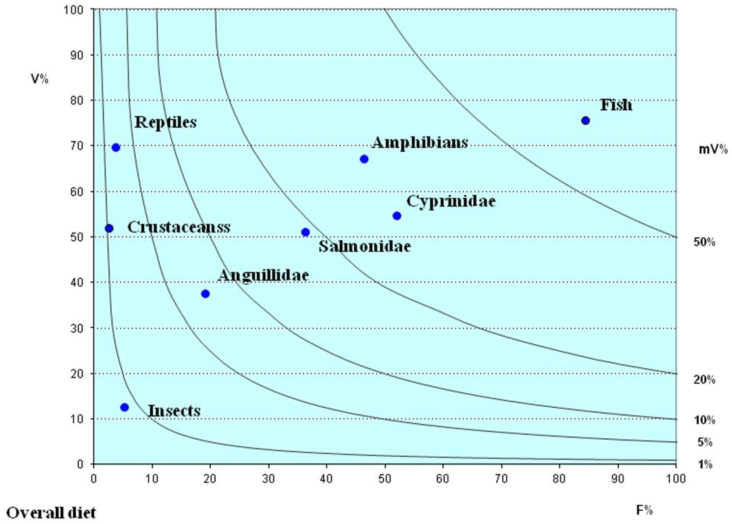

Fig. 2. Overall otter diet on the Sila Massif. Isopleths connect points of equal percent mean volume in the diet (mV%, see methods).

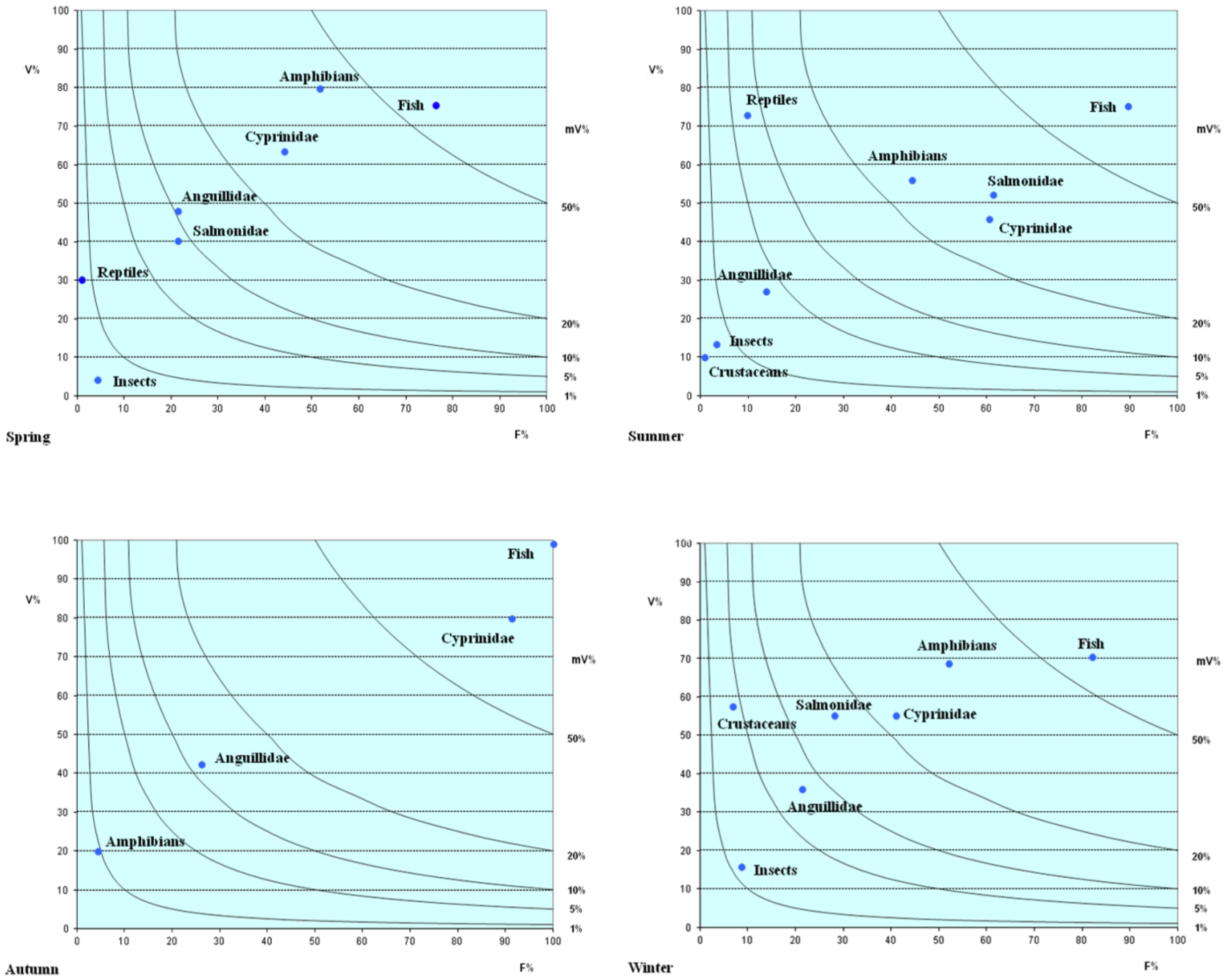

Fig3 Seasonal diet of the otter on the Sila Massif. Isopleths connect points of equal percent mean volume in the diet (mV%, see methods).

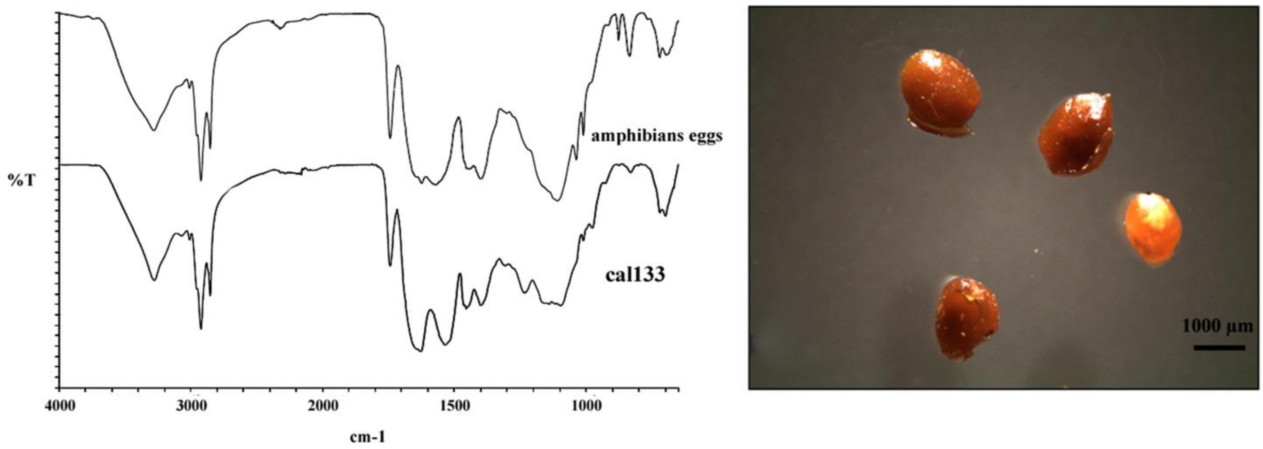

Fig4 Spectra comparing amphibian eggs to materials (sample cal133) found in otter spraints on Sila Massif (%T = trasmittance; cm-1 = Frequency). Materials found in otter spraints and suspected to be amphibian eggs (right side).

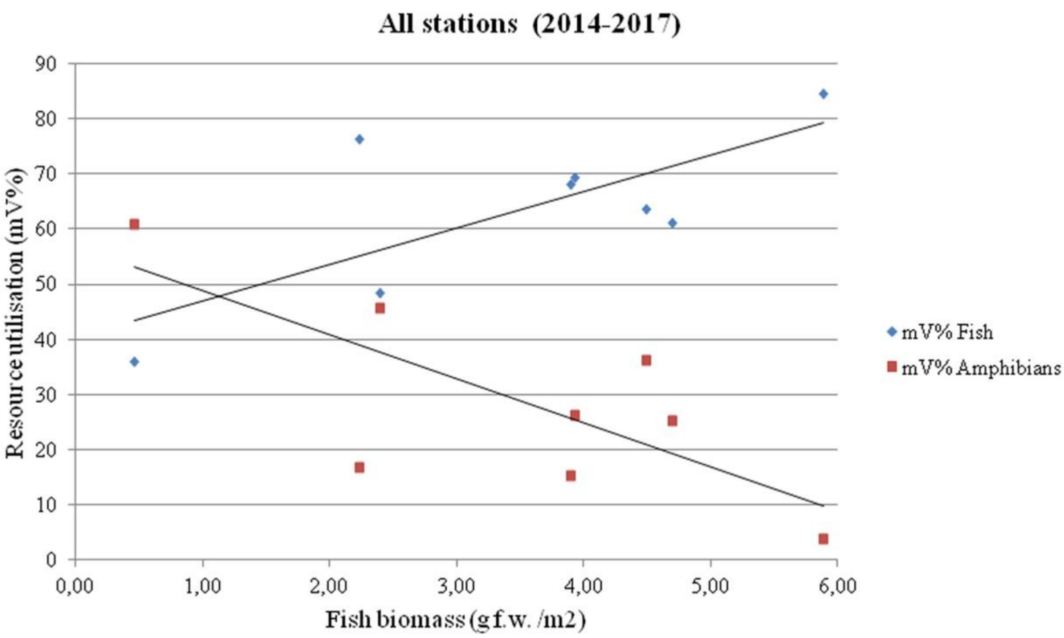

Fig5 Fish and amphibian consumption by otters related to fish biomass.

5. Amphibians in Eurasian otter diet: osteological identification unveils hidden prey richness and male-biased predation on anurans

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Amphibians in Eurasian otter *Lutra lutra* diet: osteological identification unveils hidden prey richness and male-biased predation on anurans

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ABSTRACT

1. Amphibians form a major component of otter *Lutra lutra* diet in several areas of its wide range. Yet, amphibian remains are rarely identified to species level and therefore information on the diversity of this food resource is generally scarce.

2. The aims of this study were i) to assess the overall pattern and trends in the use of this resource by otters at range-scale, and ii) highlight current knowledge on amphibian prey diversity. Additionally, we carried out the osteological identification of amphibian remains in otter spraints from S Italy, with the aim of demonstrating how it may improve our knowledge on predator-prey relationships.

3. The frequency of occurrence of amphibians in 64 diet studies averaged 12%. Otter predation on amphibians increased with longitude and was the highest in the Alpine biogeographical region. Otter predation was reported on 26 amphibian species (32% of European species). Peaks in their frequency of use were reported for all seasons, mostly in winter/spring. In S Italy, we identified 355 individuals belonging to at least seven amphibian taxa (63.6% of available species), and pointed out male-biased predation on anurans.

4. We highlighted that the contribution of amphibians to the richness of otter prey-community is by far higher than commonly perceived. Osteological identification of amphibian remains should be a major goal of diet studies, at least in the Mediterranean region, where their diversity is the highest.

Keywords: diet, geographical trends, *Lutra lutra*, prey diversity, secondary food resources.

Running head: Amphibian diversity in otter diet

Word count: 9621

INTRODUCTION

Predator–prey interactions play a major role in shaping community composition and dynamics at local scales and can shape species richness at global-scale in combination with environmental gradients and interspecific competition (Sandom et al. 2013, Terborgh 2015).

Although terrestrial mammal predators are in general highly specialized, as meaning that they can be often easily assigned to one out of seven broad diet category (Pineda-Munoz & Alroy 2014), only a minority are obligate specialists, i.e. rely on a single or handful of food resources, while most usually integrate preferred food items with a number of locally and seasonally varying "secondary resources".

A positive effect of prey diversity on predator stability has been firstly hypothesized by MacArthur (1955), who envisaged that diversity increases the reliability of the resource pool on which each predator depends. Further mechanisms that enhance predator stability when several prey species are available have been revised by Petchey (2000) and, briefly, include increased total prey biomass, lowered temporal variation in food availability, and increased opportunities to combine complementary foods to achieve a balanced nutrient intake. Diversity of the prey community may also lower resource competition, as food partitioning increases with increasing prey diversity (Sánchez-Hernández et al. 2017).

Assessing the nutritional requirements of a predator and its position along the specialist-generalist continuum is essential for reconstructing food webs and understanding its function in the ecosystem (Remonti et al. 2016). Regrettably, the possibility of investigating in details the diversity of species preyed on by elusive mammalian carnivores is often hindered by the difficulty in identifying prey remains to species level through the analysis of the undigested remains found in either faeces or, to a lesser extent, stomachs (Britton et al. 2006). Low levels of prey identification can lead to unpredictable biases in the estimate of major food-niche and community parameters of mammalian carnivores (Greene and Jaksić, 1983).

The Eurasian otter *Lutra lutra* is a top-predator of freshwaters habitats (Chanin 2003, Miranda et al. 2008, Prenda et al. 2006, Ruiz-Olmo & Jimenez 2009, Clavero et al. 2010, Almeida et al. 2012). During the 20th century, this mustelid declined dramatically throughout Europe, mainly due to water pollution, poaching, increased road traffic, and habitat loss (Prigioni et al. 2007). In Italy, the otter is currently recovering (Balestrieri et al. 2016), but it is still classified as "endangered" according to IUCN criteria (Panzacchi et al. 2010).

The semi-aquatic lifestyle of otters is metabolically costly compared to other mammals of similar sizes (Pfeiffer & Culik 1998, Kruuk 2006). Because of this, otters are ravening predators, but with the most restricted trophic niche in the mustelid family (Jedrzejewska et al. 2001, McDonald 2002). The bulk of otter diet is formed by fish, which can constitute up to 95% of their prey (Ruiz-Olmo & Palazon 1997). Nonetheless, in most areas otters also rely on alternative prey, mainly amphibians, crabs and crayfish (Mason & Macdonald 1986, Ruiz-Olmo & Palazón 1997, Jedrzejewska et al. 2001, Clavero et al. 2003, Prigioni et al. 2006, Remonti et al. 2008, 2009, Smiroldo et al. 2009), and, occasionally, birds (Mason & Macdonald 1986, de la Hey 2008). Habitat type and stability (Jedrzejewska et al. 2001, Smiroldo et al. 2009, Lanszki et al. 2016) and, ultimately, fish availability (Remonti et al. 2008) are the main factors determining the diversity of otter diet.

Amphibian remains are frequently recorded in otter spraints (e.g. Weber 1990, Brezeziński et al. 1993, Parry et al. 2011) and in several localities they form a major portion of its diet, particularly during late winter, when the availability of fish prey is reduced (Carss 1990), or in spring, when anurans aggregate in high densities to spawn (Clavero et al. 2005). In Poland (Brzezinski et al. 1993, Jedrzjewska et al. 2001, Pagacz & Witczuk 2010) and Belarus (Sidorovich et al. 1998) frogs have been reported to outweigh fish in otter diet.

This notwithstanding and unlike fish, amphibian remains are rarely assigned to species level and therefore information on the diversity of this important alternative resource is generally scarce (but see Clavero et al. 2005 and Parry et al. 2015). Although several previous reviews have analysed range-scale variability in otter diet, they mainly focused on fish prey and the effects of variation in its use or availability on the consumption of secondary food resources (Jedrzjewska et al. 2001, Clavero et al. 2003, Balestrieri et al. 2013, Krawczyk et al. 2016, Lanszki et al. 2016). Following on in Ruiz-Olmo's (1995) review on otter predation on reptiles, we reviewed available literature with the aim of outlining the level of detail currently applied to the class Amphibia in otter diet studies and the overall pattern of use of this resource by otters throughout their European range.

Italy has the highest amphibian biodiversity in Europe (43 species, of which 15 endemics; Sindaco et al. 2006, Sillero et al. 2014) and amphibians are the main otter's alternative food resource in freshwater ecosystems of the Italian otter range (Prigioni et al. 2006, Remonti et al. 2008, Smiroldo et al. 2009), their consumption being usually higher than the average for the Mediterranean region (Remonti et al. 2009). Thus, southern Italy may represent an area of choice for investigating amphibian prey diversity in otter diet. For these reasons, we applied a comparative osteological approach usually used for palaeontological and zooarchaeological studies with the aim of identifying species and, whenever possible, gender and age of the amphibians preyed on by otters on the Sila Massif (Calabria region, S Italy).

METHODS

Analysis of literature data

To assess the occurrence of amphibians in otter diet, data were collated from available literature in May and June 2018, searching the ISI Web of Science and Google Scholar online databases (key words: 'otter', 'lutra', 'diet', 'food habits'). The lists of references from downloaded articles were used to find further studies with the aim of reviewing as many articles of interest as possible.

To standardise the comparison of results from different geographical areas, data were selected according to the following criteria: (i) studies covered at least one year and were based on the analysis of spraints only, so as to avoid differences in food type representation due to differential digestion (Balestrieri et al. 2011), collected in freshwater systems; (ii) spraint sample sizes had to be greater than 100 to distinguish moderate effect sizes (Trites & Joy 2005); and (iii) diet composition had to be expressed as percent relative frequency (%RF = number of occurrences of each prey item ⁄ total number of occurrences of all prey items x 100) or could be derived from values or graphs. Results for several streams from the same area were pooled to avoid pseudoreplication (Hulbert 1984). When only seasonal data were reported, mean annual %RF was calculated from raw data when available.

Although %RF does not provide any information about the biomass or relative volume of each prey item, this index has the advantage of having been used frequently in inter-population dietary comparisons (e.g. Reynolds & Aebischer 1991, Zhou et al. 2011), and particularly in otter diet studies (Balestrieri et al. 2013, Krawczyk et al. 2016, Lanszki et al. 2016), for which %RF values have been shown to be nearly as accurate as other indices (Jacobsen & Hansen 1996).

When geographical coordinates of the study area were not indicated, they were derived from ordinance survey maps, with representative mean coordinates used when samples were collected over a large area. Geodetic coordinates were projected to Eastings and Northings using a Transverse Mercator projection. Study areas were then grouped according to biogeographical regions. Spearman's correlations between geographical coordinates and arcsine transformed (Zar 1984) RFs of amphibians were calculated. Seasonal variation in otter predation on amphibians was tested by the chi-square test (χ^2), using raw frequency data.

Case study: osteological identification of amphibians

Spraints were collected in a ca. 2040 km^2 large area of Southern Italy ("Sila Piccola" massif, Calabria region), as part of the long-term monitoring of a recently rediscovered otter population (see Gariano & Balestrieri 2018). Otters occurred on six rivers for a total of 12 sampling stations and 357 spraints, of which 91 collected in summer 2014 and 2015 and 266 in 2016-2017 (summer: 34; autumn: 30; winter: 85; spring: 117).

Undigested remains were sorted according to standard methodologies (e.g. Smiroldo et al. 2009), and the bones of amphibians and reptiles were identified using a comparative osteological approach. Taxonomic identification was based on available keys (e.g., Haller-Probst & Schleich 1994 and Ratnikov & Litvinchuk 2007, 2009, for caudates; Bailon 1999, for anurans; Fig. 1, 2) and reference collections (see Appendix S1 for a brief description of the most significant diagnostic features).

Bailon's diagnostic keys (1999) are based on French anurans and do not allow to discriminate between *R. italica* and *R. dalmatina*, both currently occurring on the Sila massif. Consequently, most brown frog remains were attributed to *R. dalmatina/italica*. Nonetheless, based on our observations on available comparative material, the frontoparietals of *R. italica* (Fig. 2, A-C) show a wide and deep groove on the posterolateral part of their dorsal surface which does not occur in *R. dalmatina* (Fig. 2, D-E). This feature allowed to distinguish the two species whenever we found well-preserved frontoparietals.

When identification to species level by only osteological criteria was not possible (e.g. for *Lissotriton* spp. and *Pelophylax* spp.), a biogeographical rationale was also applied, assigning the remains to the only species currently present in the study area. Whenever possible also the age (juveniles vs. adults) and sex of aech specimen were assessed. Age identification was mainly based on the size and degree of ossification of the bones. The sex of adult (large sized) Ranidae and Bufonidae was assessed on the basis of the sexually dimorphic mesial crest of the humerus (more robust in males than in females; Bailon 1999).

Diagnostic bones were photographed using a Leica M205 microscope equipped with the Leica application suite V 4.10. This apparatus allows taking multiple pictures of a single specimen at different focus planes ("Z-stacks"). The different pictures are then merged by the image acquisition software to create a new, virtual picture showing the specimen entirely in focus.

The chi-squared- and Kruskal-Wallis' tests were used to test for seasonal variation in, respectively, the raw frequencies of amphibians in otter diet and mean number of individuals per spraint.

RESULTS

Amphibians in otter diet

Sixty-four studies, published between 1969 and 2017, met our criteria (Tab. 1). They were carried out in 20 countries, from Sweden in the north to Greece in the south and from Portugal in the west to Lithuania in the east (Fig. 3). Percent RF of amphibians in otter diet ranged between 0 in NE Spain (Melero et al. 2008) to 43 in Poland (Pagacz & Witczuk 2010), averaging (±SE) 12 ± 3.2 (Fig. 4). In most studies, amphibians accounted for less than 15% of otter diet (Fig. 5). No latitudinal trend could be outlined, while otter predation on amphibians increased with longitude (Spearman's rho = 0.47, $P < 0.001$), and from the Atlantic and Mediterranean biogeographical regions (mean %RF = 8.9 and 11.2) to the Alpine region (23.25), with the Pannonian, Continental and Boreal regions showing intermediate values (12.3-14.2).

Information on seasonal variation in otter diet was available for 34 studies. Otters preyed on amphibians mostly in winter (52.9%) and/or spring (67.6%), but peaks in their frequency of use were reported also for summer and autumn (14.7% and 20.6%, respectively; $\chi^2 = 23.5$, 3 d.f., $P < 0.001$).

Only a minority of reports (17%) classified amphibian prey at species-level: the number of species recorded ranged between 1 and 5 (mean = 3.0). Considering all available studies consulted for this review and our own results (see below), till now 26 amphibian species have been reported to be preyed on by otters (Tab. 2), of which 8 Salamandridae, 3 Discoglossidae, 2 Pelodytidae, 2 Bufonidae, 3 Hylidae and 8 Ranidae. Four species (*Lissotriton boscai*, *Discoglossus galganoi, Pelodytes ibericus* and *Rana iberica*) are endemic to the Iberian peninsula and three (*Lissotriton italicus, Hyla intermedia* and *Rana italica*) to Italy (Sindaco et al. 2006, Speybroeck et al. 2016).

Amphibians preyed on by otters in southern Italy

Amphibian bones were found in 159 spraints (45.1%), allowing the identification of a minimum of 352 individuals (2.2 individuals/spraint, min-max: 1-21; Tab. 3), belonging to at least seven amphibian taxa (*Rana italica*, *Rana dalmatina*/*italica, Pelophylax* kl. *bergeri/hispanicus, Hyla intermedia, Bufo bufo, Bufotes balearicus* and *Lissotriton italicus*). Most bone remains belonged to the Ranidae family (273 individuals, 76.9%), mainly either agile- or Italian stream frogs (*Rana* *dalmatina/italica*, 168 individuals). Only three well-preserved frontoparietals were found, which belonged to two different individuals of *R. italica*. Green frogs (either edible frogs or pool frogs, *Pelophylax kl. bergeri/hispanicus*) were preyed on less frequently (24 individuals). Twenty-seven individuals belonged to Bufonidae (7.3%), of which ten were assigned to European green toad *Bufotes balearicus* and four to common toad *Bufo bufo*. Hylidae were represented by the Italian tree frog *Hyla intermedia* (six individuals, 1.7%). Urodelans included three Italian newts (*Lissotriton italicus;* 1%). Based on the size and degree of ossification of the remains, 330 preyed amphibians were adults and 22 juveniles. In total, sex determination was possible for 31.5% of the adults, 85 males and 19 females corresponding to a male:female ratio of 4.5:1.

The frequency of amphibian remains in otter diet varied seasonally (Tab. 4), being the lowest in autumn when frogs were recorded in otter spraints only once ($\chi^2 = 24.7$, 3 d.f., $P < 0.001$). From winter to summer the mean number of individuals/spraint kept constant (1.7-2.0; $\chi^2 = 0.78$, 2 d.f., P = n.s.)

DISCUSSION

Currently otters have been recorded to prey on 28 amphibian species, corresponding to 34.5% of all species available throughout their European range (Speybroeck et al. 2016). These numbers, which are certainly underestimated, highlight the opportunistic feeding behaviour of the Eurasian otter, which, although having been shaped by evolution as a fish-specialist predator, is able to shift to a large variety of semi-aquatic species when fish availability is low (Clavero et al. 2004, Remonti et al. 2008, Smiroldo et al. 2009).

Unexpectedly, with 8 species (31%) urodelans consistently contributed to the diversity of otter diet. In the 1980s, Chanin (1985) suggested that either newts are not predated by otters or they are missed through standard spraint analysis. In the last decade, newt remains have been recovered in the stomachs of otter carcasses both in SW England (*Triturus cristatus*; Britton et al. 2006) and Hungary (*Lissotriton vulgaris*; Lanszky et al. 2015), while newts have been recorded in otter spraints in Wales (*Lissotriton helveticus*; Parry et al. 2015) and the Czech-Moravian Highlands (Poledník et al. 2007). The negligible percentage of spraint-based studies reporting newts may depend on the difficulty in identifying amphibian remains (Parry et al. 2015).

Further evidence has been obtained by the direct observation of otters preying on *Triturus cristatus* in North Jutland (Bringsøe & Nørgaard 2018) and *Pleurodeles waltl* in S Spain (Cogălniceanu et al. 2010). In the latter area, otters ate only the internal organs of ribbed newts, a behaviour that may lead to the underestimation of their contribution to the mustelid’s diet (Cogălniceanu et al. 2010).

Although at different concentrations depending on the species (Hanifin 2010), newts are known to produce tetrodotoxin, a powerful anti-predatory, skin secretion which may explain why otters do not eat newts whole. Otherwise, otters may avoid the ingestion of poison by skinning them, a behaviour that has been described for toads in Portugal (Beja 1996), Spain (Lizana & Pérez Mellado 1990), Belarus (Sidorovich & Pikulik 1997), Finland (Sulkava 1996) and Wales (Slater 2002). As reported for toads (Lizana & Pérez Mellado 1990), the large amount of time spent for handling newts may explain otter's general preference for frogs, but recent observations (Cogălniceanu et al. 2010) suggest that local availability may also play a major role.

Previous reviews focused on the geographical variability in otter diet. Mason and Macdonald (1986) reported that in southern Europe otters generally eat more amphibians and reptiles than in the north, and Adrian and Delibes (1987) suggested that the frequency of occurrence of amphibians, reptiles and insects in otter sprains decreases as latitude increases. In contrast, Jedrzejewska et al. (2001) did not find any latitudinal pattern in otter diet in Eurasia, while Clavero et al. (2003) did not include coastal otters in their analyses and found that otters inhabiting Mediterranean localities had more diverse diets than those in northern regions. In any case, both studies pointed out that habitat features (especially water availability) are important factors influencing the diet of otters. More recently, Balestrieri et al. (2013) found no relationship between dietary breadth and latitude, suggesting that habitat-related variations in fish assemblage richness and stability play a major role in shaping otter diet. Accordingly, otters prey on fish more frequently in standing waters surrounded by riparian vegetation than in waters flowing in open habitats (Krawczyk et al. 2016) and fish consumption decreases with stream elevation (Remonti et al. 2009).

In agreement with previous studies (Jedrzejewska et al. 2001, Balestrieri et al. 2013), we could not highlight any latitudinal trend, while the frequency of amphibians in otter diet increased with longitude, with the highest %RFs recorded in the eastern Alpine- (Poland and Bulgaria) and Boreal (Finland, Belarus) regions (see also Lanszki et al. 2016). As suggested by Kruuk (2006) this trend may depend on the availability of large numbers of frogs and toads in those regions. Despite this general trend, we recorded a wide variation in amphibian contribution to otter diet, e.g. in southern Italy %RF ranged between 7.2 and 33.7, suggesting that it is shaped by the local availability of prey species, mainly fish (Remonti et al. 2008, Smiroldo et al. in press).

While the abundance of anuran populations may be the largest in north-eastern Europe, the richness of amphibians communities is the highest in Mediterranean Europe (Sillero et al. 2014), as confirmed by the relatively large number of endemisms preyed on by otters in the Iberian and Italian peninsulas. Therefore, to unveil the actual diversity of otter prey, in south-western Europe the detailed analysis of bone remains should be a primary concern.

This task is time-consuming and, despite the availability of several useful keys, requires a certain degree of lab experience. For this reason, and because research often focused on fish prey, only few of the selected studies reported sufficient information on the diversity of amphibian prey in otter diet. In our study area, osteological identification of amphibian remains allowed to ascertain otter predation on seven out of the 11 species recorded for the study area (63.6%; Sindaco et al. 2006). Three – Italian newt *Lissotriton italicus*, Italian tree frog *Hyla intermedia* and Italian stream frog *Rana italica* - are endemic to the Italian peninsula and are listed as Low Concern in the Italian Red List of threatened species (Rondinini et al. 2013). The discrimination of *Rana italica* from *Rana dalmatina* was allowed by a newly described diagnostic feature, based on the morphology of frontoparietals, which unfortunately, were seldom preserved in conditions as good as to allow species identification. Mean number of amphibian individuals per spraint (2.2) was higher than that reported by the only previous study which attempted to assess numbers of otter prey (1.6; Clavero et al. 2005). As recorded throughout Europe, frogs formed the bulk of the amphibian fraction of otter diet.

While, according to literature data, the frequency of otter predation on amphibians is usually the highest in late winter and spring, when they aggregate in large numbers to spawn, in our study area amphibian remains occurred in otter spraints throughout the year, with the exception of autumn, when otters mainly preyed on cyprinids (Smiroldo et al. in press). As frogs are often preyed on during spawning, it is hard to discriminate whether seasonal diet shifts depend on their availability or fish shortage (Kruuk 2006), nonetheless, in Mediterranean habitats, predation on amphibians is generally inversely related to fish biomass (Remonti et al. 2009, Novais et al. 2010, Krawczyk et al. 2016; but see Ayres and García 2011 for an opposite opinion). Most brown frogs recovered in otter spraints in summer probably belonged to widespread *Rana italica*, which is more strictly associated to freshwater habitats throughout the year with respect to *R. dalmatina* (Romano et al. 2012) and in summer is mainly crepuscular and nocturnal (Sindaco et al. 2006). Accordingly, in the study area the Italian stream frog has been reported to be by far more widespread than the agile frog (41.4% vs. 8.0% of 36 sampling stations; Montillo 2017).

The analysis of bone remains allowed to point out male-biased predation by otters on anurans. Sex biased mortality induced by predation is a general phenomenon, with males being often the sex paying the heaviest toll (Christe et al. 2006). Otters as predators are not an exception to this general rule: male Atlantic salmons (*Salmo salar*) have been reported to be killed by otters more frequently than females (Carss et al. 1990). As for fish, male-biased predation on anurans is likely to depend on the breeding behaviour of prey. First, the total number of males at breeding sites greatly exceeds that of females (Merrell 1968, Calef 1973), as females leave the pond soon after having laid their eggs (Hartel et al. 2007), while males stay close to breeding sites longer in order to mate with more females. Moreover, during the breeding season advertisement calls are mainly given by males, exposing them to a greater risk of being detected by predators. Although we do not know to which extent mustelids rely on auditory cues to find their prey, European polecats have been reported to take large numbers of male agile frogs during their spawning season (Lodé 1996). Juveniles were preyed on only in spring and summer, when their availability is the highest, but in very low numbers, suggesting that otters prefer the most profitable adult individuals.

CONCLUSIONS

Our results show that, although amphibians generally represent a secondary food resource with respect to fish, their contribution to the richness of its prey-community is higher than commonly perceived, and osteological analysis allow to investigate to a deeper extent the feeding behaviour of this top-predator of freshwater habitats. Throughout its wide range the otter can rely on amphibians wherever fish availability is insufficient and thus this resource may play a major role in otter ongoing recovery in parts of its range where freshwaters have been deeply altered by human activities (Smiroldo et al. in press).

Conversely, amphibians are among the species of highest conservation concern due to the dramatic decline affecting their populations at global-scale (Wake & Vredenburg 2008, Blaustein et al. 2011). This worldwide decline in amphibian populations might threaten the viability of otters living in freshwater systems that have been depleted of their fish communities. In turn, otter expansion may affect the survival of small populations of endemic amphibian species, as otter are able to kill several individuals in a very short period of time (Cogălniceanu et al. 2010, Parry et al. 2015). Further studies are needed to understand the potential role played by otters in amphibian conservation and detailed knowledge of predator-prey interactions represent a first step in that direction.

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Table 1. Location, study period, number of analysed otter spraints (N) and percent relative frequency (%RF) of amphibians in otter diet for 64 European study areas (Biogeographicale regions: Boreal, Mediterranean, Atlantic, Continental, Alpine, Pannonian).

Code	References	Country	Bio-Region	Period	Latitude	Longitude	N	Classification level	%RF
1	Baltrunaite 2009	Lithuania	6-BOR	2006-07	55°17'N	23°55'E	1593	Amphibians	3.4
2	Beja 1996	Portugal	9-MED	1991-92	37°38'N	08°39'W	2883	Frogs-toads	9.6
3	Bonesi et al. 2004	England	4-ATL	1998-99	50°36'N	03°41'W	161	Amphibians	4
4	Bouros & Murariu 2017	Romania	7-CON	2012-14	45°43'N	27°00’E	233	Species	20.2
5	Britton et al. 2017	England	4-ATL	2014-15	51°03′N	01°48′E	140	Amphibians	10
6	Brzeziński et al. 2006	Poland	7-CON	1 year	54°00’N	23°00’E	1202	Amphibians	11.5
7			7-CON	1 year	50°20'N	22°40'E	513	Amphibians	12.4
8			7-CON	1 year	53°00’N	23°00’E	1374	Amphibians	12.9
9			1-ALP	1 year	49°20'N	22°40'E	950	Amphibians	27.2
10	Bueno-Enciso et al. 2014	Spain	9-MED	2009-10	39°11’N	04°15’W	731	1 species	2.17
11	Callejo & Delibes 1987	Spain	9-MED	1974-79	42°45'N	03°30'W	367	1 species	0.5
12	Carss et al. 1990	Scotland	4-ATL	1987-88	57°01'N	03°15'W	324	Amphibians	2.9
13	Chanin 1981	England	4-ATL	1972-73	50°16'N	03°39'W	389	Amphibians	0.5
14			4-ATL		50°36'N	03°41'W	253	Amphibians	0.6
15	Clavero et al. 2004	Spain	9-MED	2001-02	36°00’N	05°36'W	1682	Amphibians	6.5
16	Delibes et al. 2000	Spain	9-MED	1981-82	37°00’N	06°26'W	307	Amphibians	10.7
17	Erlinge 1969	Sweden	6-BOR	1966-68	58°00’N	15°15'E	350	Amphibians	1.6
18	Freitas et al. 2007	Portugal	9-MED	2003-04	38°02'N	8°22'W	1680	Amphibians	2.03
19	Fusillo et al. 2003	Italy	9-MED	2001	40°16'N	15°22'E	564	species	33.73
20	Geidezis 2002	Germany	7-CON	1994-96	51°20'N	14°58’E	1099	Amphibians	2.5
21	Georgiev 2006	Bulgaria	1-ALP	2005-06	42°40'N	25°32'E	1155	species	9.4
22	Harna 1993	Poland	1-ALP	1987-89	49°21'N	22°04'E	379	species	14.5
23	Jedrzejewska et al. 2001	Poland	7-CON	1988-96	52°41'N	23°53'E	396	species	11.61
24	Karamanlidis et al 2013	Greece	9-MED	2010-12	40°36′N	21°33′E	414	Amphibians	29
25	Kemenes & Nechay 1990	Hungary	11-PAN	1989	47°17'N	19°13'E	270	Amphibians	0.82
26	Kloskowski et al. 2013	Poland	7-CON	1997-98	51°03’N	23°00’E	478	Amphibians	10.7
27	Knollseisen 1995	Austria	7-CON	1992-93	49°00’N	15°00’E	175	Amphibians	3.1
28	Kortan et al. 2010	Czech Rep.	7-CON	2003-04	50°52'N	14°14'E	349	Amphibians	2.9
29	Kyne et al. 1989	Ireland	4-ATL	1984-86	53°33'N	07°21'W	2349	Amphibians	15
30	Lanszki & Kormendi 1996	Hungary	11-PAN	1992	46°24'N	17°59'E	873	Amphibians	9.9
31	Lanszki & Molnar 2003	Hungary	11-PAN	1996-98	46°14’N	17°29’E	801	Amphibians	4.3

32			7-CON	1999-01	46°18’N	16°52’E	116	Anura	18.3
33			11-PAN	2001-02	46°44’N	17°45’E	234	Anura	34.3
34	Libois & Rosoux 1991	France	4-ATL	1982-87	46°16'N	00°43'W	165	Amphibians	9.85
35	Libois 1997	France	7-CON	1991	45°06'N	02°40'E	704	Amphibians	9.66
36	Lopez-Nieves & Hernando 1984	Spain	9-MED	1979	38°22'N	03°49'W	2145	1 species	10
37	Marques et al. 2007	Portugal	9-MED	2003-05	40°17'N	06°57'W	206	species	11.56
38	Melero et al. 2008	Spain	9-MED	2002-05	41°49’N	01°53’E	108	-	0
39	Miranda et al. 2008	England	4-ATL	2004-05	51°22'N	02°52'W	358	Amphibians	1.96
40	Morales et al. 2004	Spain	9-MED	1997	40°00’N	07°00’W	426	species	6.2
41	Ottino & Giller 2004	Ireland	4-ATL	1996	52°12’N	08°06’W	287	Anura	17.1
42	Pagacz & Witczuk 2010	Poland	1-ALP	2008-09	49°08'N	22°40'E	284	Amphibians	43
43			7-CON		49°38’N	18°43’E	400	Anura	15.4
44	Polednik et al. 2004	Czech Rep.	7-CON	2000-02	49°38’N	18°43’E	136	Anura	17.3
45			7-CON		49°35’N	18°45’E	358	Anura	30
46			9-MED		42°40'N	11°35'E	122	Rana spp.	7.6
47	Prigioni et al. 1991	Italy	9-MED	1987-88	40°35'N	16°25'E	461	Rana spp.	9.1
48			9-MED		42°40'N	11°35'E	148	Rana spp.	10.2
49			9-MED		40°10'N	16°10'E	490	Rana spp.	12
50	Prigioni et al. 2006	Italy	9-MED	1996-97	40°30'N	15°30'E	193	Amphibians	7.2
51			9-MED	2001-03	40°16'N	16°00'E	555	Amphibians	17.5
52	Remonti et al. 2008	Italy	9-MED	2001	40°00'N	16°30'E	1323	Amphibians	23.9
53			9-MED		42°00’N	00°00’E	610	Amphibians	1.67
54	Ruiz-Olmo & Palazon 1997	Spain	9-MED	1984-96	42°00’N	00°00’E	755	Amphibians	4.8
55			9-MED		42°00’N	00°00’E	1432	Amphibians	6.16
56			9-MED		42°00’N	00°00’E	596	Amphibians	9.32
57	Sales-Luis et al. 2007	Portugal	9-MED	1996-97	40°21'N	08°09'W	1328	species	3.8
58	Sidorovich et al. 1998	Belarus	6-BOR	1988-95	56°00’N	32°00’E	641	Amphibians	35.6
59	Smiroldo et al. 2009	Italy	9-MED	2006	40°16'N	16°00'E	838	Amphibians	35
60	Sulkava 1996	Finland	6-BOR	1988-93	62°15'N	24°25'E	1506	Amphibians	16.5
61	Taastrom & Jacobsen 1999	Denmark	4-ATL	1990-91	56°20'N	09°06'E	587	Amphibians	2.3
62			4-ATL		56°20'N	09°06'E	391	Amphibians	19.26
63	Weber 1990	Scotland	4-ATL	1987	57°08'N	02°05'W	919	Amphibians	23.5
64	Smiroldo et al. in press.	Italy	9-MED	2014-17	39°20’N	16°40’E	357	Amphibians	21.6

Table 2. Amphibian species preyed on by otters as assessed by reviewing available literature.

Species	Country	References	
Triturus marmoratus	Portugal	40, 57	
Triturus cristatus	Denmark, England		Britton et al. 2006; Bringsøe & Nørgaard 2018
Lissotriton vulgaris	Hungary		Lanszki et al. 2015
Lissotriton italicus	Italy	this study	
Lissotriton helveticus	Wales		Parry et al. 2015; Polednik et al. 2007
L. (Triturus) boscai	Portugal	57	
Pleurodeles waltl	Spain		Cogălniceanu et al. 2010
S. salamandra	Portugal, Spain	37, 40	
Discoglossus galganoi	Spain		Romero & Guitian, 2017
Bombina bombina	Hungary		Lanszki et al. 2015
Bombina variegata	Bulgaria, Poland	21, 22	
Pelodytes ibericus	Spain		Clavero et al. 2005
Pelodytes cultripes	Spain		Clavero et al. 2005, Cogălniceanu et al. 2010
Bufo bufo	Belarus, Bulgaria, England, Finland, France, Hungary, Italy, Poland, Portugal, Scotland, Spain	21, 23, 57,this study	Beja 1996; Lodé 1996; Sulkava 1996; Sidorovich & Pikulik 1997; Lanszki et al. 2001; McCafferty 2005; Ayres & García 2007; Parry et al. 2011
Bufotes viridis	Bulgaria,	21	
Bufotes balearicus	Italy	this study	
Hyla intermedia	Italy	this study	
Hyla meridionalis	Spain		Clavero et al. 2005
Hyla arborea	Romania, Hungary	4	Lanszki et al. 2009
Pelophylax lessonae	England		Forman et al. 2012
P. (Rana) perezi	Portugal, Spain	10, 11, 40, 57	
P. (Rana) esculenta	Hungary, Italy, Poland, Romania	4, 19, 23	Lanszki et al. 2015
P. (Rana) ridibunda	Bulgaria, Hungary, Romania, Spain	4, 21, 36	Lanszki et al. 2001
P. bergeri/hispanicus	Italy	this study	
Rana temporaria	England, Italy, Poland, Romania, Scotland	4, 19, 22, 23	Forman et al. 2012; McCafferty 2005
Rana dalmatina	Bulgaria, France, Hungary, Italy, Romania	4, 21, this study	Lodé 1996; Lanszki et al. 2001
Rana iberica	Portugal, Spain	40, 57	
Rana italica	Italy	this study	

Table 3. Number of adults, juveniles, females and males of amphibians preyed on by the otter in the rivers of the Sila Massif (N_{tot}: total number of spraints; N_{amp}: number of spraints containing amphibian remains; a.s.l.: above sea level).

River	Station	m a.s.l.	Ntot	Namp.	Species	Adults	Juveniles	♀	♂
Savuto	Parenti	720	*24*	*10*	*Rana dalmatina/italica*	16	-	1	7
					Ranidae indet.	2	-	-	-
					Bufonidae indet	1	-	-	-
					Anura indet	2	-	-	-
	Balzata	560	*51*	*39*	*Rana dalmatina/italica*	68	-	2	34
					P. kl. *bergeri/hispanicus*	2	-	-	2
					Hyla intermedia	2	-	-	-
					Ranidae indet.	24	1	3	1
					Anura indet.	9	-	-	1
	Altilia	168	*44*	*16*	*Rana dalmatina/italica*	7	-	-	5
					P. kl. *bergeri/hispanicus*	3	-	-	-
					Ranidae indet.	14	-	3	-
					Bufonidae indet	3	-	-	-
					Anura indet	5	-	-	-
	San Mango	85	*27*	*15*	*Rana dalmatina/italica*	9	-	-	5
					P. kl. *bergeri/hispanicus*	1	-	-	2
					Ranidae indet.	12	-	2	-
					Bufo bufo	1	-	-	-
					Bufonidae indet	3	-	-	-
					Anura indet	3	-	-	-
Tacina	downstream	764	*9*	*4*	*Rana dalmatina/italica*	6	4	2	1
					Hyla intermedia	1	-	-	-
Lese	upstream	180	*38*	*19*	*Rana dalmatina/italica*	17	-	2	13
					P. kl. *bergeri/hispanicus*	2	-	-	-
					Hyla intermedia	1	-	-	-
					Ranidae indet.	7	2	1	-
					Bufo bufo	1	-	-	-
					Bufotes balearicus	3	-	-	-
					Bufonidae indet	2	-	-	-
					Anura indet	6	1	-	-
	downstrem	140	*134*	*55*	*Rana dalmatina/italica*	18	9	1	6
					P. kl. *bergeri/hispanicus*	12	3	-	1
					Hyla intermedia	2	-	-	-
					Ranidae indet.	17	1	1	-
					Bufo bufo	2	-	-	-
					Bufotes balearicus	6	1	-	-
					Bufonidae indet	4	-	1	-
					Lissotriton italicus	3	-	-	-
					Anura indet	16	-	-	-
Neto	upstream	173	*15*	*1*	*Rana dalmatina/italica*	1	-	-	1
Amato	downstream	520	*14*	*6*	*Rana dalmatina/italica*	13	-	-	6
					P. kl. *bergeri/hispanicus*	1	-	-	-
					Ranidae indet.	1	-	-	-
					Anura indet	1	-	-	-
Total			356	165		330	22	19	85

Table 4 - Seasonal distribution of the number of adults, juveniles, females and males of amphibians found in otter spraints from the Sila Massif.

Species	**Summer**					**Autumn**					**Winter**					**Spring**					**Year**
	Adult	**Juv.**	♀	♂	**tot**	**Adult**	**Juv.**	♀	♂	**tot**	**Adult**	**Juv.**	♀	♂	**tot**	**Adult**	**Juv.**	♀	♂	**tot**	
R. dalmatina/italica	54	9	5	22	63	-	-	-	-	0	46	-	1	20	46	55	4	3	36	59	168
P. kl. *berg/hispan*	8	2	-	1	10	-	-	-	-	0	4	-	-	-	4	9	1	-	1	10	24
Ranidae indet.	25	2	1	-	27	1	-	-	1	1	31	-	5	-	31	20	2	4	-	22	81
Hyla intermedia	6	-	-	-	6	-	-	-	-	0	-	-	-	-	0	-	-	-	-	0	6
Bufo bufo	-	-	-	-	0	-	-	-	-	0	1	-	-	-	1	3	-	-	-	3	4
Bufotes balearicus	5	-	-	-	5	-	-	-	-	0	-	-	-	-	0	4	1	-	-	5	10
Bufonidae indet	-	-	-	-	0	-	-	-	-	0	-	-	-	-	0	13	-	1	-	13	13
Lissotriton italicus	2	-	-	-	2	-	-	-	-	0	-	-	-	-	0	1	-	-	-	1	3
Anura indet	14	-	-	-	14	-	-	-	-	0	13	-	-	-	13	15	1	-	-	16	43
Total	114	13	6	23	127	1	0	0	1	1	95	0	6	20	95	120	9	8	37	129	352

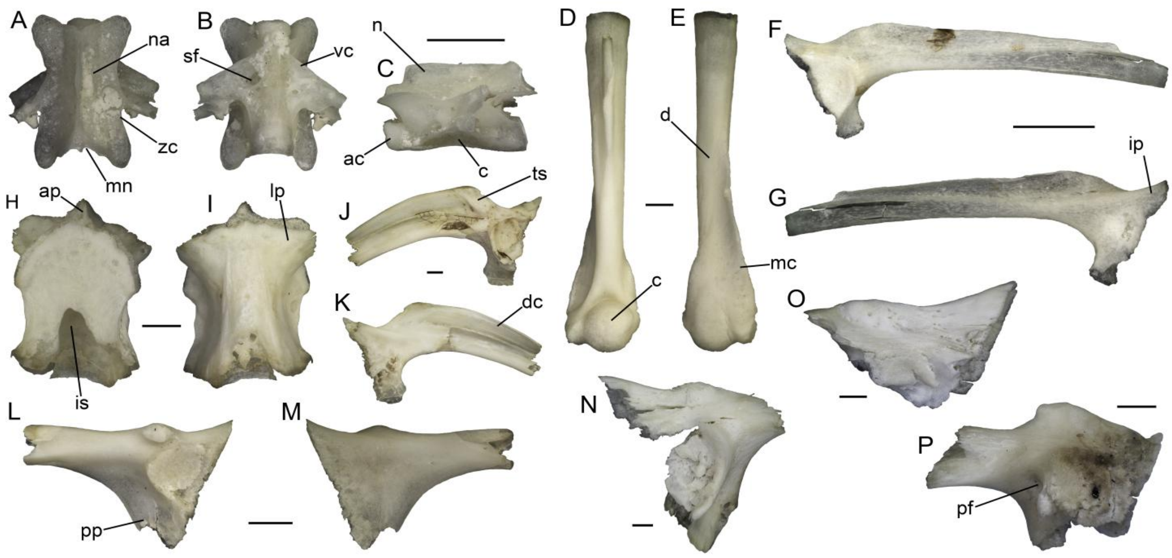

Figure 1. Trunk vertebra of *Lissotriton italicus* in dorsal (A), ventral (B) and left lateral (C) views; left humerus of *Rana dalmatina/italica* in ventral (D) and dorsal (E) views; right ilium of a juvenile *R. dalmatina/italica* in lateral (F) and medial (G) views; sphenetmoid of *Pelophylax* spp. in dorsal (H) and ventral (I) views; left ilium of *Pelophylax* spp. in lateral (J) and medial (K) views; left ilium of *Hyla intermedia* in lateral (L) and medial (M) views; right ilium of *Bufo bufo* in lateral view (N); left ilium of *B. bufo* in lateral view (O); left ilium of *Bufotes* sp. in lateral view (P). Abbreviations: ac, anterior condyle; ap, anterior process; c, centrum; d, diaphysis; dc, dorsal crest; ip, ischiadic process; is, incisura semielliptica; lp, lateral process; mc, medial crest; mn, median notch; n, neurapophysis; na, neural arch; pf, preacetabular fossa; pp, pubic process; sf, subcentral foramina; ts, tuber superior; vc, ventral crest; zc, zygapophyseal crest. Scale bars equal 1 mm.

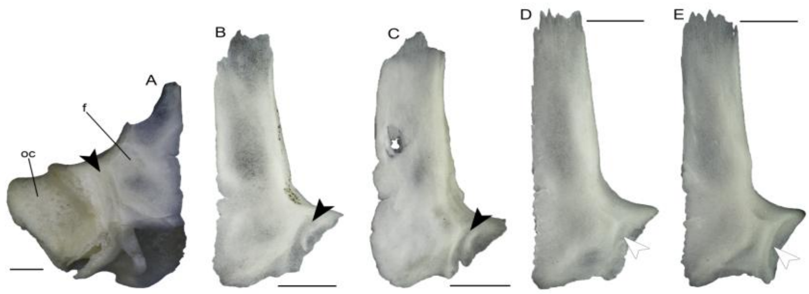

Figure 2. Fragmentary left frontoparietal of *Rana italica* fused with the otic complex (A) in dorsal view; dorsal view of right frontoparietals of *R. italica* (B, C), and *R. dalmatina* (D, E). Abbreviations: f, frontoparietal; oc, otooccipital complex. Scale bars equal 1 mm. Black arrows mark the groove on the frontoparietals of *R. italica*, whereas white arrows mark its absence in *R. dalmatina*.

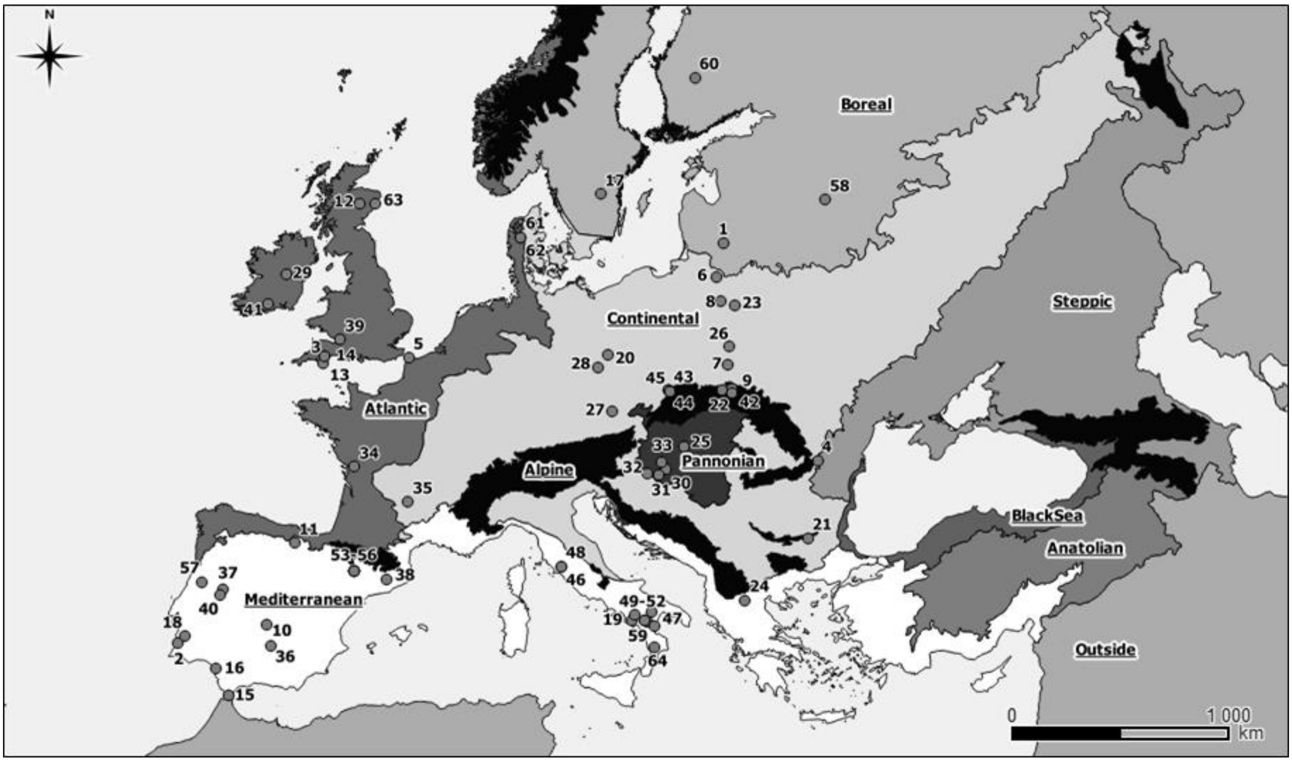

Figure 3. Distribution of the 64 reviewed European studies on otter diet (numbers correspond to those in Table 1). Biogeographical regions are shown.

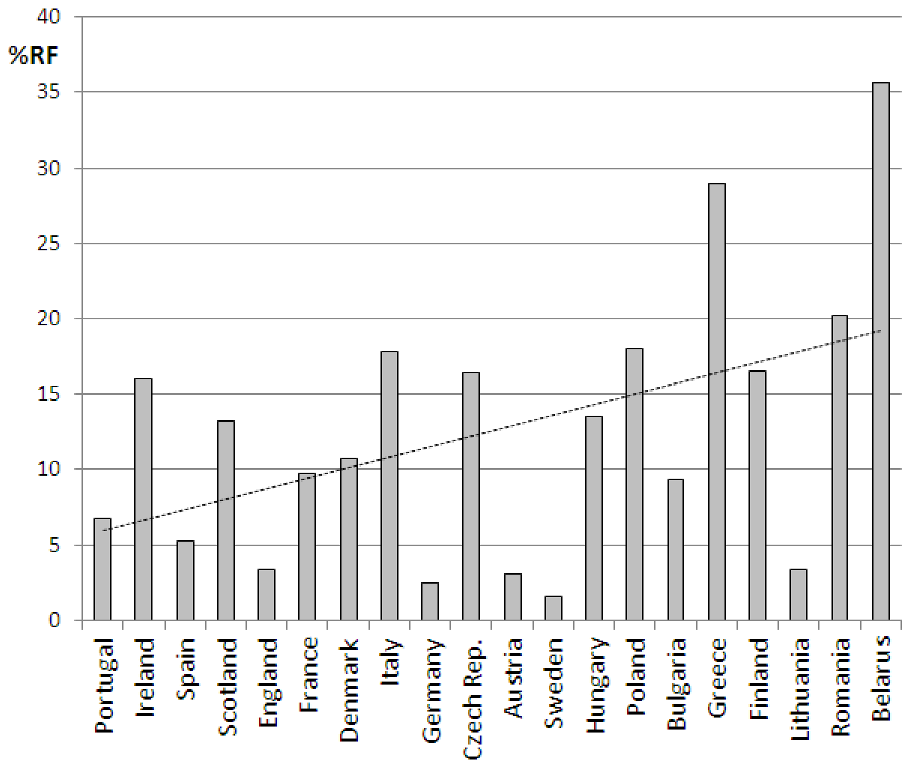

Figure 4. Country-level variation in the mean percent relative frequency (%RF) of amphibians in otter diet. Countries are listed from west to east, based on the longitude of their capitals.

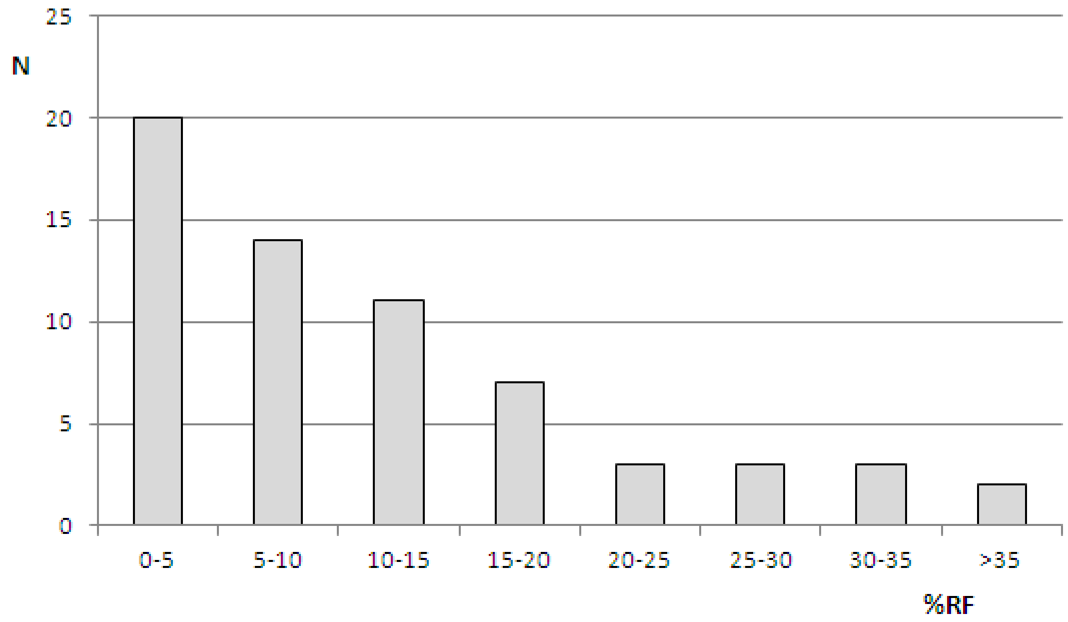

Figure 5. Number (N) of studies on otter diet per class of percent relative frequency of occurrence (%RF) of amphibians.

Appendix S1

Identification of bone remains

We provide a brief report of some of the most significant diagnostic features used to identify the osteological remains of amphibians and reptiles in otter spraints. For a complete account, the reader is referred to the references listed in the methods section and below.

Small and elongated trunk vertebrae with an opisthocoelous centrum, subcentral foramina on the ventral surface, and a median notch on the posterior margin of the neural arch clearly belong to a small-sized urodelan (Ratnikov & Litvinchuk 2007). The anteroventrally-inclined anterior condyle, the high neural arch, the well-developed posterior median notch, the high neurapophysis with a horizontal dorsal margin, and the well-developed ventral and zygapophyseal crests allow the assignment of these vertebrae to the genus *Lissotriton* (Ratnikov & Litvinchuk 2007; Fig. 1, A-C). *L. italicus* is the only species of the genus known to occur in the study area (Sindaco et al. 2006; Sillero et al. 2014).

The ilium is one of the most taxonomically useful bones of the anuran skeleton (e.g., Sanchiz 1998, Blain et al. 2015, Gómez & Turazzini 2015). Those belonging to brown (*Rana* spp.) and green (*Pelophylax* spp.) frogs are characterized by a dorsal crest (Bailon 1999). In *Rana* spp., the crest shows a poorly distinct tuber superior, forming a very wide angle with the ischiadic process of the ilium (Fig. 1, F-G), whilst the tuber superior is well-distinct and forms a ca. 90° angle in *Pelophylax* spp. (Fig. 1, J-K). Several other bones can be attributed with certainty to either brown- or green frogs, out of which we usually found well-preserved humeri and sphenethmoids. Humeri of male brown frogs (Fig. 1, D-E) have a straight and thin diaphysis, with no paraventral crest, a distal condyle that is located by the main axis of the bone, and a well-developed medial crest which is longer than in green frogs and bends in dorsal direction (Bailon 1999). The sphenethmoid of green frogs (Fig. 1, H-I) can be identified based on the moderate anteroposterior elongation, no dorsoventral compression and sella amplificans, moderately deep incisura semielliptica and short anterior and lateral processes, which in ventral view are more distinguishable than those of brown frogs (Bailon 1999).

The frontoparietals of *R. italica* (Fig. 2, A-C) show a wide and deep groove on the posterolateral part of their dorsal surface which does not occur in *R. dalmatina* (Fig. 2, D-E). This feature allowed to distinguish the two species whenever we found well-preserved frontoparietals.

Ilia without a dorsal crest mainly differ in the morphology of their tuber superior. Those with a globular and laterally bending tuber and expanded pubic process belong to *Hyla* spp. (Bailon 1999; Fig. 2, L-M), of which only *H. intermedia* occurs in the study area (Sindaco et al. 2006). Large and tough ilia with a dorsally rounded tuber superior pertain to *Bufo bufo* (Bailon 1999; Fig. 2, N-O). A bilobed tuber and deep preacetabular fossa are typical of the ilia of *Bufotes* spp. (Bailon 1999; Fig. 2, P).

Snakes are usually identified through the morphology of their vertebrae. Elongated trunk vertebrae with a sigmoid and rounded on the back hypapophysis, a neural arch vaulted on the back, and robust parapophyseal processes belong to *Natrix natrix* (Szyndlar 1984; Fig. S1, A-E; six adults and three juveniles/subadults were found in spraints from the River Lese). Large and elongated trunk vertebrae with a ventral hemal keel, which is flattened and widens to the rear, can be attributed to *Hierophis viridiflavus* s.l. (Szyndlar 1984, 1991a; Fig. S1, F-G; two individuals in as many spraints from the rivers Tacina and Simeri). As the populations of this species occurring in southern Italy have been recently attributed to *Hierophis carbonarius* (Mezzasalma 2015*),* the remains found in otter spraints were assigned to the latter species.

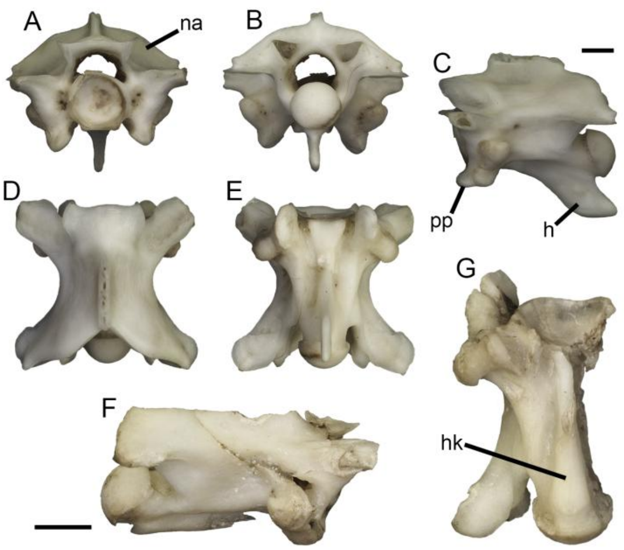

Figure S1. Trunk vertebra of *Natrix natrix* in anterior (A), posterior (B), left lateral (C), dorsal (D) and ventral (E) views; trunk vertebra of *Hierophis carbonarius* in right lateral (F) and ventral (G) views. Abbreviations: h, hypapophysis; hk, hemal keel; na, neural arch; pp, parapophyseal process. Scale bars equal 1 mm.

6. Effects of the presence of native river otter and environmental features on a southern Italian amphibian communities on Sila Massif

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Ecoscience 2018 (*submitted*)

Effects of the presence of native river otter and environmental features on a southern Italian amphibian communities on Sila Massif.

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ABSTRACT

Different studies show that alien mammal predator can contribute to amphibian populations decline. On the contrary studies on the impact of autochthonous mammals are extremely scarce. Here we assessed the relative role of Eurasian otter (*Lutra lutra*) on amphibian breeding community in a Mediterranean area, in eight streams with different habitat features. In 2017 88 stations, on Sila Massif (southern Apennines, Italy) were surveyed for amphibian breeding, recording, otter marking activity and fish presence.. We detected the breeding of seven species: *Salamandra salamandra*, *Salamandrina terdigitata*, *Bufo bufo*, *Hyla intermedia*, *Pelophylax* kl. *berg/hispan*, *Rana dalmatina*, and *Rana italica*. Stream features explained a significant amount variation of community structure. The composition of amphibian communities was strongly affected by illuminance and slope diversification. While no negative relationship was recorded between otter abundance and amphibians breeding despite, their role in the otter diet; *P.* kl. *berg*/*hispan* showed significant positive relationship with otter abundance. Statistical analyses didn't show any negative impact on amphibian community and furnish useful insights for conservation projects aiming to restore native *L. lutra* populations throughout their past native range.

Keywords

River otter, predation impact, endangered species, native,amphibian community , illuminance.

Word count: 5253

INTRODUCTION

Riverine ecosystems represent the 0.006% of the world's freshwaters (Shiklomanov 1993), their biological value contain a rich and varied biota, including a high diversity of plants, algae vertebrate and invertebrates, many of which remain undiscovered. The strong human pressure on freshwater ecosystems has a deep influence with negative ecological effects at both local and regional scales; these effects will be irreversible especially for damp biotopes so that freshwater communities are considered the most threatened on Earth (Malmqvist and Rundle 2002). Mediterranean basin is one of the most breakable areas, which freshwater habitats host a huge number of endemic species (Myers et al. 2000; Olson and Dinerstein 2002; Abell et al. 2008) especially for amphibians, reptiles and invertebrates (Lanza 1983; Manenti et al 2018). Nowadays the current loss of biodiversity, have been termed the "sixth mass extinction" (Wake and Vredenburg 2008; Barnosky et al. 2011), and amphibians are the most threatened vertebrates, with an estimated 40% of amphibian species currently in danger of extinction (Bishop et al. 2012; Ficetola et al 2015). Their decline represents the most dramatic example of vertebrate extinction that is currently taking place (Wake and Vredenburg, 2008). Habitat destruction, pollution, exotic species introductions, disease, overexploitation, climate change and UV radiation and emerging infectious diseases (i.e. *Batrachochytrium dendrobatidis*) are the main causes of amphibians decline (Reading 2007; Wake 2007; Lips et al. 2008; Rohr et al. 2008; Ribeiro et al. 2009). In the Mediterranean area, and especially in Italian Apennines, habitat loss and climate change resulted to be major causes of recent population decline (D'Amen & Bombi 2009; D'Amen et al. 2010). In addition, the introduction of allochthonous species as the freshwater crayfish *Procambarus clarkii* may threat amphibians' occurrence (Ficetola et al 2011). While the role played by alien predator species in affecting amphibians distribution is currently a wide studied argument, few data are available on the role played by mammal native predator species that are declining and that may be the object of future conservation plans. One of the mammal amphibian predator is the river otter (*Lutra lutra*) (Jedrzejewska et al 2001; Clavero et al 2003); this species is a freshwater top predator that suffered a severe decline in Europe during the 20th century. To avoid the risk of extinction, it was introduced in the Appendix 1 of CITES, Appendix II of the Bern Convention (Council of Europe, 1979) and Annexes II and IV of the 'EC Habitats & Species Directive' (92/43/EEC). In Europe this mustelid is classified as 'Near threatened' by the IUCN, while in Italy it is classified as "Endangered" (EN) and it is listed in the national Red List of endangered species, according to IUCN criteria (IUCN 2001). Otters have the most restricted trophic niche in the mustelid family (Jedrzejewska et al 2001, McDonald 2002) and, it's mainly piscivorous if the fish supply is not limiting (Ruiz-Olmo et al.

1989; Prigioni et al. 1991; Remonti et al. 2008), but, on the other hands, otters are able to show a flexible feeding behavior with a strong interest versus amphibian as major alternative resource (Smiroldo et al 2009; Almeida et al. 2012; Balestrieri et al. 2013). The impacts of autochthonous otter, on population sizes of amphibians are poorly known (Lodè et al 2004), contrary to other mammal predators not native such as American mink (*Mustela vison*) (Ahola et al. 2006; Salo et al., 2010). In this paper we want to assess the role played by native otter populations on the amphibians' communities considering stations with and without otter in the Southern Apennines. In particular, we aim to: i) verify the relative role of different stream features and ii) to assess if otter occurrence may be detrimental for amphibians breeding.

MATERIALS AND METHODS

Study area

Amphibian, fish and otter monitoring was performed in eight rivers from Sila Massif (South Italy): Savuto and Amato Rivers flowing into the Tyrrhenian Sea; Corace, Alli Simeri, Crocchio and Tacina Rivers, which flows southwards into the Squillace Gulf (Ionian Sea); in the east Neto River, with Lese River as its main tributary (Fig. 1).

Amphibian monitoring

Amphibians surveys were performed during spring 2017 on 88 sampling sites belonging to the eight watercourses described above. In each site, two surveyors used visual encounter surveys (Crump and Scott 1994) to evaluate the presence/absence of breeding adults, eggs or tadpoles of amphibians. We repeated the samplings two times for each site. To maximize the homogeneity of monitoring among streams and pools, the same observers performed all surveys for all sites. Each site was formed by a linear transect (of ca. 150 m) along streams. Damp biotopes were described on the basis of rapid bioassessment protocol (Barbour et al. 1999). We recorded the main features describing stream morphology, quality and ecosystem functioning, that can be important for the reproduction of amphibians. We measured: 1) substrate heterogeneity; 2) slope heterogeneity; 3) illuminance grade; 4) stream width; 5) maximum depth. Substrate heterogeneity was evaluated on the basis of the percentage of alternation of substrate elements (sand, gravel, stones, sunken branches, see Petersen, 1992). Each site or stream transect was classified using the following rank scale: 1, absence of diversification, only a single substrate element covering almost 100% of the site; 2, poorly diversified, only 2 substrate elements covering >90% of the transect; 3, quite diversified, at least three elements present in at least 10% of the transect; 4, highly diversified, >90% of the transect presenting an alternation of at least three elements. Slope heterogeneity was measured similarly to substrate diversification: 1, absence of diversification, only a single element covering almost 100% of the slope; 2, poorly diversified, only 2 elements covering >90% of the slope; 3, quite diversified, at least three elements present in at least 10% of the slope; 4, highly diversified, >90% of the transect presenting an alternation of at least three elements along the slope. Elements included also decaying woods. Illuminance grade was measured using a PCE EM882 luxmeter (illuminance, measured in lux, accuracy 0.01 lux) by performing at least 10 measures of illuminance in the portions of the sector receiving more light..

Otter and fish monitoring

Otter and fish monitoring was performed in the same sites and time of amphibians surveys. Otter occurrence was assessed by recording along each transect the number of spraints (feces). The survey of feces ("spraints") was performed following the 'Standard Method' recommended by the IUCN/SSC Otter Specialist Group (Reuther et al. 2000): during each survey all shoreline areas of each transect were searched by walking on both riversides and around small islands and looking for typical otter sprainting sites (e.g. large stones, bridges, pool banks, confluences), according to Macdonald and Mason (1983) and Prigioni (1997). Details of the monitored stations and the results of the overall otter monitoring in the area are reported in Gariano and Balestrieri (2018). In this work, only data referred to the same period and the same stations of the amphibian monitoring were considered (Spring 2017). According to literature, the intensity of the otter marking activity, measured as the number of spraints over 100 m of shoreline (n° of spraints/100 m) can be taken as a measure of otter abundance. n° of spraints/100 m are calculated dividing the total number of spraints recorded in each station, in each survey, divided by the ratio between the length of the station (m) and 100 m.

Fish monitoring was performed by electrofishing, which usually provides an effective estimate of fish relative abundance at lower costs than removal sampling (Fièvet et al. 1999; Ross et al. 2001). Details of the apparatus and the sampling modalities are reported in Smiroldo et al. (submitted). Fish monitoring was performed in the same station and time than the amphibian monitoring, and thus is fully comparable. Briefly, electrofishing revealed the occurrence of 7 species, 5 were autoctonous: *Salmo trutta, Leuciscus cephalus, Rutilus rubilio, Anguilla Anguilla and Cobitis taenia, and 2 alloctonous: Carassius carassius and Gambusia affinis*. Trout was the only fish species at middle-high-altitude (generally above 400 m a.s.l.), on the rivers Simeri, Tacina, Alli, Amato, Savuto and Corace, while cyprinids, mainly chub and roach, predominated below 200 m a.s.l. In this work, fish community was considered as a dichotomous variable (present or absent).

Statistical analyses

A site is surely "occupied" if a species is detected at that site, but not detecting a species during sampling does not necessarily indicate the species is absent (MacKenzie, D.I. 2006). We used Presence 5.5 to calculate the per sampling detection probability of each species, using the observation history of the two different samplings performed for each site and assuming a constant detection probability across surveys that were performed in the same season. We then calculated survey reliability as the probability of detecting a species after two surveys, following the formula in (Gomez-Rodriguez et al 2012). We used a constrained redundancy analysis (RDA) to evaluate the relative role of stream features on the multivariate structure (i.e., species composition) of amphibian communities. RDA is a canonical analysis, merging the features of regression and ordination techniques, that permits to estimate how much of the variation of the structure of one dataset (e.g., community composition in a wetland; endogenous dataset) is explained by independent variables (e.g., habitat features; exogenous datasets) (Borcard, 2011).We considered one matrix of stream features including the biotic and abiotic variables recorded and we used the matrix of amphibians recorded as the endogenous dataset. As detection probability was high and the cases of failing to record species were very low (<0.05%), data was recorded for each site on the presence/absence of each species. We used variance partitioning to calculate the independent and joint effect of stream features; we calculated the significance of explained variance by performing ANOVA-like permutation tests (10,000 permutations) (Borcard, 2011). To assess if otter occurrence may be detrimental for amphibians breeding occurrence we built for each amphibian species a generalized linear mixed effects model (GLMM) which allows taking into account random factors determining non-independence of observations (Pinheiro and Bates 2000). For each species binomial GLMMs were used with breeding occurrence as dependent variable and otter spraints number as independent variable. Random factor was the main stream of belonging of each site in order to account for the possible variation linked to the catchment basin. All analyses were performed under the R 3.4.1 environment using the packages lmerTest, car and vegan (Pinheiro and Bates 2000).

RESULTS

For amphibian species, two surveys allowed to assess absence with high reliability. Detectability of amphibians among the sites was generally high (> 0.9) and for the species which showed occurrence values > 7 % the failure to detect species was <0.05%. We observed the breeding of seven species, such as *Salamandra salamandra* (occurrence, O = 1 %), *Salamandrina terdigitata* (O = 2.2 %), *Bufo bufo* (O = 30.3 %), *Hyla intermedia* (O = 3.4 %), *Pelophylax* kl. *berg*/*hispan* (O = 12.3 %), *Rana dalmatina* (O= 7.8 %) and *Rana italica* (O= 25.9 %).

Fish were recorded in 33 sites of the amphibian monitoring (37.5 %), while otter spraints were detected in 61 sites (69.3 %). RDA analysis shown in figure 2 assessed the relationship between 5 environmental variables (steam depth and width, illuminance and slope and substrate diversification), fish occurence and otter abundance to amphibian species, excluding those with occurrence < 7 %. Community structure was significantly related to stream features ($P < 0.01$). The effect of stream features explained 15 % of the variation. The first RDA axis (Fig. 2) was represented by sites 'illuminance'. The 'otter abundance' together with 'slopes diversification' represented mainly the second RDA axis (Fig. 2), while 'fish occurrence', stream 'depth' and 'substrate diversification' showed a lower effect on amphibian community. *R. italica* was linked to sites with high illuminance, while *Bufo bufo* preferred wide 'width' with lower 'slope differentiation' and *P.* kl *berg*/*hispan* showed a positive link with sites with high 'otter abundance'.

GLMM analysis highlighted that the abundance of otter spraints generally did not negatively affect the breeding of the species recorded (Table 1), and in one case, the breeding of *P.* kl. *berg*/*hispan*, we recorded a positive relationship between otter presence and the breeding of this species..

DISCUSSION

Stream features affected the distribution of breeding sites of most amphibians in the study area, and the main role was played by sites illuminance. In Mediterranean area characterized by strong irregular river flow, species must metamorphose before their habitat dries (Blaustein et al. 2001), indeed many amphibians lay eggs in open shallow areas where they receive maximum exposure to sunlight, this exposure can heat egg masses, which induces fast hatching and developmental rates (Stebbins and Cohen 1995). In particular *R. italica* and *B. bufo* showed a positive relationship with sites with high illuminance rate. *R. italica* is mainly nocturnal (Baran and Atatür, 1997; Vignoli et al.,2014), in different studies (Tattersall et al., 2006; Michaels and Preziosi, 2013) metamorphs, juveniles and adults of a number of nocturnal frog species have been observed, in the wild, basking in sunlight in diurnal retreat sites to receive reflected UV-B, indicating that even nocturnal species may rely on exposure to UV-B radiation to synthesize vitamin D3. This vitamin in most vertebrates is synthesized via exposure to the ultraviolet B radiation (UV-B) present in sunlight, its deficiency is an important nutritional problem, and plays a critical role in regulating calcium metabolism, as well as significant roles in organ development, muscle contraction and the functioning of the immune and nervous systems (Wright and Whitaker, 2001). Furthermore basking has been considered important for increases the rates of digestion, growth, fat deposition, improve the ability to escape from predators (Seymour, 1972; Lillywhite et al., 1973; Carey, 1978; Brattstrom, 1979; Huey and Stevenson, 1979; Freed, 1980; Hutchison and Dupre´, 1992; Rome et al., 1992), provides benefits to control of external parasites (Cagle 1950), and protection against bacterial infections (Kluger 1977, 1978). Slope diversification and river otter occurrence played similar role. Both slope and substrate diversification are generally important for amphibians breeding as they provide places for attaching egg-clutches and shelters for both breeding adults and larvae (Wells, 2007). However, in our case we found a negative relationship between *B. bufo* breeding and 'slope diversification', meaning that this species prefers more simple slopes with higher ease of access. In particular, this species was often found to breed in pools with rocky and poorly diversified slopes where males may be favored in detecting females.

Considering the specific role of river otter on amphibian community, particularly worthy is the fact that the GLMMs analyses, did not detect a negative relationship between river otter sprainting activity and any amphibians breeding and confirmed the positive relationship with *P.* kl. *berg/hispan*. This is a considerable aspect because in Mediterranean area amphibians are an important secondary food resource for this mammal (Remonti et al 2009; Smiroldo et al 2009). In fact, in the harsh Mediterranean freshwater ecosystems, fish biomass is often insufficient for otter and alternative prey food resources become necessary (Ruiz-Olmo et al. 2001; Remonti et al., 2008; Remonti et al., 2009; Smiroldo et al., 2009). The fact that we did not find a negative relationship between otter spraints presence and amphibians breeding is linked to the fact that amphibians in our study area have long co-evolution history with an autochthonous predator like the otter; These amphibians may have evolved antipredator responses to the otter foraging strategies evading detection through crypsis, area avoidance, seeking shelter (Lima and Dill 1990; Smith 1992). Moreover, predator–prey interactions considered that native predators do not have large detrimental effects on the population sizes of their native prey, because have coexisted with their prey for long periods, limiting or even regulating the population sizes of their prey (Gurevitch et al 2000; Korpimaki et al 2004). Particularly interesting is the positive relationship that we detect about *P.* kl. *berg/hispan* breeding and river otter spraints. Future studies will be necessary to assess if this relationship is linked to similar habitat requirements in the lower parts of rivers. Very few data are available about the effect of autochthonous mustelids abundances on amphibians use of breeding sites. Other than the river otter also the polecat *Mustela putorius* may prey upon amphibians. In France the selective predation by polecats on *Rana dalmatina* appears to change their sex ratio and promote monogamy (Lodé et al. 2004). Different is the case of introduced mammalian predators that may affect amphibians breeding and survival (Innes et al. 2010; Goldson et al. 2015). Ahola (et al 2006) show that predation by an introduced generalist mammalian predator, such as the American mink (*Mustela vison*), in island ecosystems may have drastic detrimental effects on population densities of frogs. Our study provides new information on stream ecological factors driving breeding of amphibians in lotic environments of southern Italy and reveal that the effect of native predator like the river otter may not be detrimental for the breeding sites selection. This result should be considered in conservation project aiming to restore native otter populations throughout its past native range.

DECLARATION OF INTEREST STATEMENT

The authors have no competing interests to declare.

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Table 1. Results of the binomial GLMMs performed using species breeding as dependent variable and log-transformed abundance of otter spraints as independent variable. In bold significant relationships

	B	se	χ^2	P
Bufo bufo	-0.08	0.38	0.04	0.86
Pelophylax kl. *berg/hispanicus*	1.8	0.65	8.05	**<0.01**
Rana italica	0.68	1.34	0.25	0.61
Rana dalmatina	-0.88	0.71	1.5	0.21

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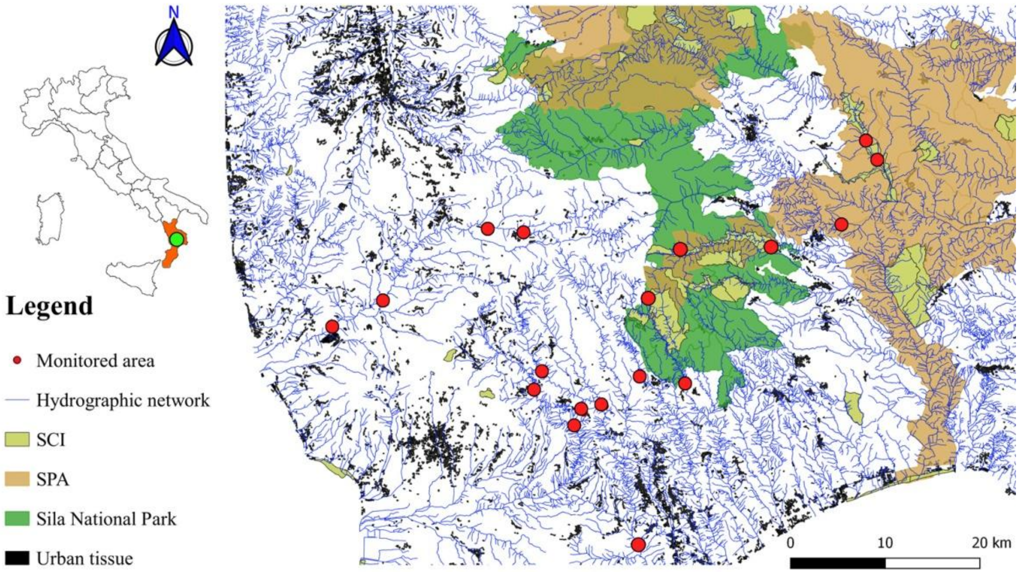

Fig 1. Monitored area (Calabria region, Southern Italy) . SCI: Site of Community Importance; SPA: Special Protection Area.

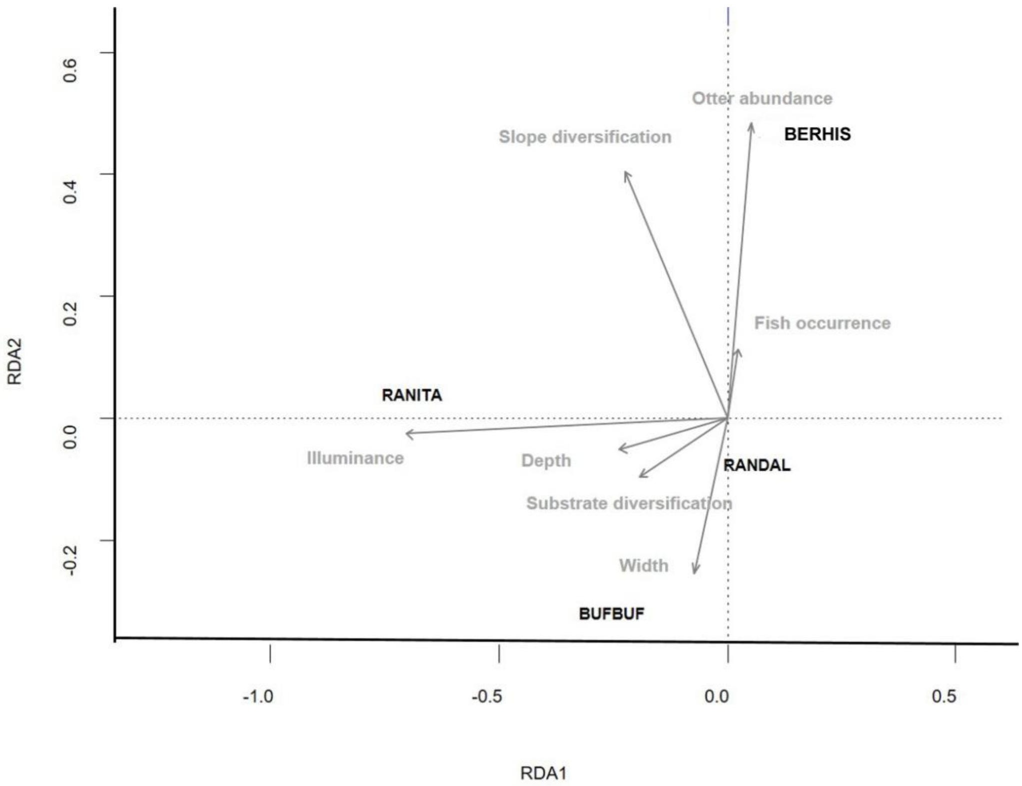

Fig 2. Results of constrained redundancy analysis showing the relation between habitat features and the distribution of amphibian species. Constraining variables are represented by grey arrows. BUFBUF, *Bufo bufo*; RANITA, *Rana italica*, RANDAL, *Rana dalmatina*; BERHYS, *Pelophylax* kl. *bergeri/hispanicus.*

7. Conclusion

River otter distribution recorded on Ticino Valley suggests an expansion of the species along this important ecological corridor. The recording river otter activity in this area is extremely important considering the link of Ticino River with Po River in the South and Lake Maggiore in the North. The low marking activity found in Ticino River in respect to other areas such as Sila Massif, suggests the presence of low number of individuals, but in the intricate fluvial system present in the Ticino valley, leaves some incertainties about the actual consistency. Genetic analyses, now in progress, will clarify the minimum number of individual in the area. Furthermore, a fascinating hypothesis, which can be tested by genetic analysis, is that the otters in the southern part of the sublacual Ticino River may derive from a small group escaped from local extinction. Introduced otters have a genetic component of the line-B, while the eventually relictus nucleous not. Anyway, it's important to carry on with the otter monitoring in the area. In light of the recent recorded signs of the otter presence in Switzerland in the upper Ticino River, could be necessary to extend the monitoring area to Lake Maggiore, the link between italy and Switzerland.

According to previous monitoring, the survey on Sila Massif showed a stable status of the otter populations in the area, with a slight positive trend supported by the finding of spraint activity on Amato River (Catanzaro). Otter presence in this river was never reported previously. In the southern Italian range, the otter distribution is still fragmented and it is necessary to facilitate the connection among populations such as through the upper Volturno (Molise region) and Calore (Campania) catchments. Habitat restoration and a stronger activity for contrasting illegal fishing may enhance existing populations increasing the connectivity between Sila Massif and the core area of Pollino Massif. The diet analysis in Sila Massif showed the great importance of amphibians as main alternative food resource, wherever fish availability is insufficient. The protection of the riparian habitat in particular of amphibians breeding sites through the institution of buffer zone around wetlands may enhance amphibian comminities, and thus increasing the ongoing recovery of otter populations. Moreover, amphibians are among the species of highest conservation concern due to the dramatic decline affecting their populations at global-scale. Data reported here exclude a negative effect of otter predation on amphibian community.

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Giorgio Smiroldo – watercolor on paper 23x16 cm (2011)
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