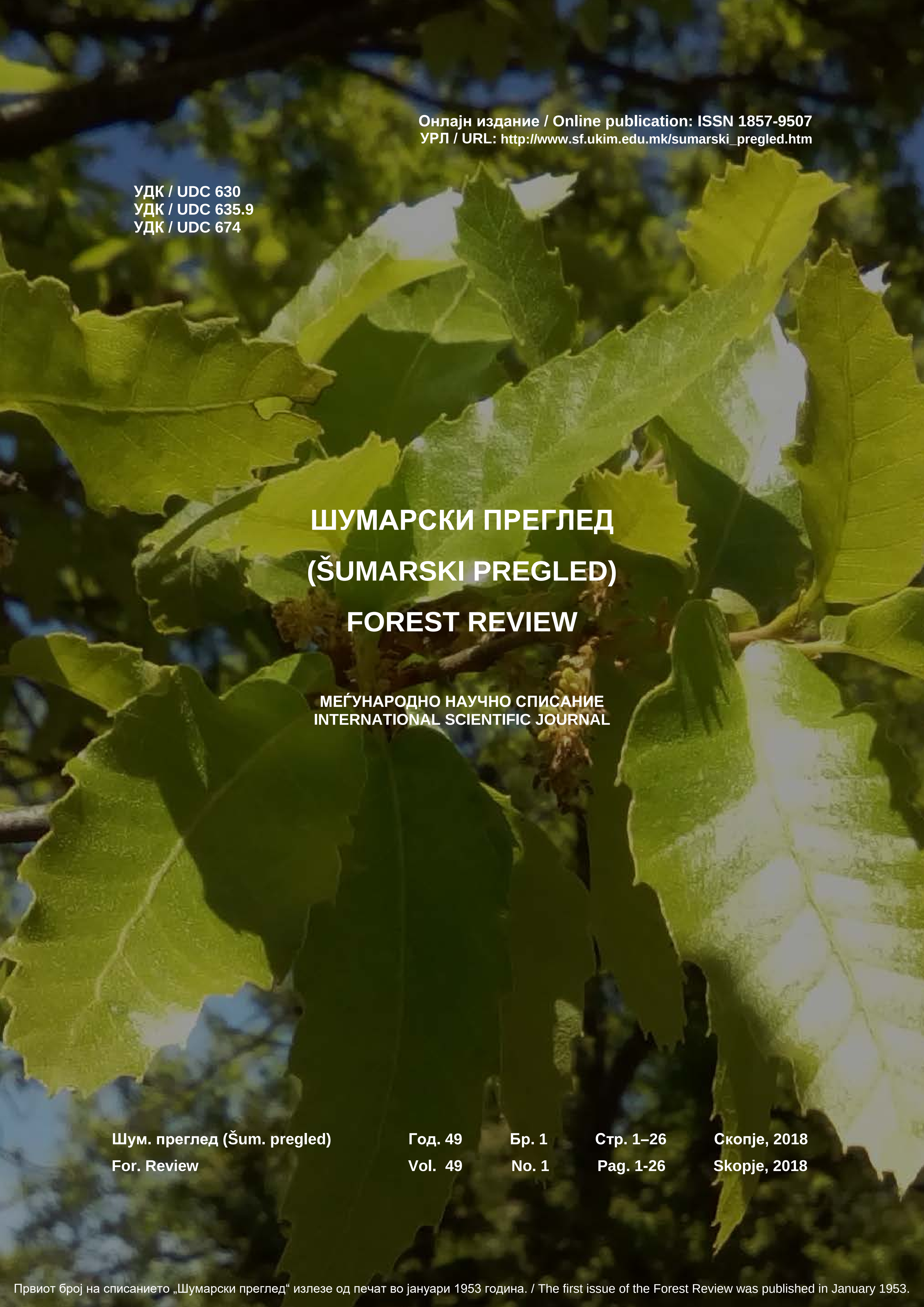

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Detecting alternative prey of the Balkan lynx using scat analysis

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ABSTRACT: The Eurasian lynx (*Lynx lynx* L. 1758) is an ambush predator with preference for medium-sized ungulates. The felid, however, has a varied diet which includes other animals, ranging from lagomorphs and rodents to chiropterans and small carnivores. The paper offers an overview of the alternative diet of the endangered population of the Balkan subspecies (*Lynx lynx* subsp. *balcanicus*) in western Macedonia. Alternative prey detection was done by analysing scat material collected near feeding site or found by chance in the period between 2010 and 2018. A total of 37 scats were retrieved. Of the total scats brought for analysis, only 28 gave conclusive results. Scat samples were analysed for morphological characteristics, undigested parts of the prey item (hairs and bones), also including immunological and serological analysis of the immunoglobulins present. Data reveals a diverse assortment of animal prey species with roe deer, chamois, brown hare, marten, wild boar, rat, mouse, red squirrel, edible dormouse, shrew, and pipistrelle bat. Such dietary shift in this predator may be owning to the unavailability of its principal prey item, namely roe deer, but also chamois. Therefore, anthropogenic pressure on the main prey species presents significant threat to ensuring a healthy and viable Balkan lynx population.
Keywords: Balkan lynx, alternative prey, scats, immunoglobulins, hairs

1 INTRODUCTION

The Eurasian lynx (*Lynx lynx* L. 1758) is a medium-sized polyphagus carnivore that evolved to live and hunt in the Palearctic forests. Its preferred prey depends on the geographical region that lynx occupies. For example the main prey in Europe are medium-sized ungulates – roe deer (*Capreolus capreolus*) and chamois (*Rupicapra rupicapra*) [15, 30, 31, 33, 25], and in the taigas of North Siberia it is lagomorphs [26]. Throughout its vast area of distribution, the Eurasian lynx is observed to prey also on red deer (*Cervus elaphus*), reindeer (*Rangifer tarandus*), fallow deer (*Dama dama*), muntjac (*Muntiacus* sp.), sika deer (*Cervus nippon*), lagomorphs (brown hare – *Lepus europaeus*), rodents and shrews (dormice, voles, mice, squirrels), birds (tetraonids, passerines), small carnivores (red fox – *Vulpes vulpes*, mustelids, wildcat – *Felis silvestris*, raccoon dog – *Nyctereutes procyonoides*), wild boar (*Sus scrofa*), amphibians and insects [6, 13, 24, 35, 20, 32]. Although rarely, domestic animals, like sheep and goats, enter the lynx diet [19, 32]. Lynx prey variation and selection depends on many factors, but the most important are habitat, prey availability, sex and age, and season [47]. Larger prey is mostly consumed during spring–summer, and predominantly by adult males, while smaller prey, alternative prey included, is more typical of autumn–winter predation in adult females with kittens and in subadults [35]. Alternative prey might be important for the survival of lynx in their early years [35] or of nurturing mothers [17, 22]. It is noted by Jobin *et al.* [17] that lynx prey upon roe deer and chamois according to their abundance. Lynx are quite adaptive to food regime of feast and famine; when enough prey, males on average consume 3.4 kg meat per night [17], but in between two larger kills they can survive several days on minimum or no food at all. Alternative prey becomes important during intermediate periods (between two large kills) or in low availability of main prey, due to poaching, for example. This poses an important issue in view of its hunting strategy—lynx are ambush predators—since its constant presence at a given area tends to alert the prey. Therefore, lynx require "large and exclusive home ranges to ensure constant hunting success" [4], which necessitates stable and somewhat evenly distributed prey population. Due to the disturbance in the Macedonian forests, mainly from legal or illegal logging, poaching, hunting, stray dogs, etc., lynx main prey (roe deer) is disturbed and vigilant [48, 49, 50]. This makes it hard to kill, so we assume that lynx, up to some extent, are forced to depend on alternative prey. Lynx enlarge their home range when the main prey becomes less available or its population number drops down critically [51]. Okarma *et al.* [35] noted that the decline in roe deer was followed by decline in lynx population. The use of alternative prey often depends on the relative abundance of the preferred prey [9]. Some studies suggested that Eurasian lynx shift their diet to the alternative prey when the preferred prey is greatly reduced [10, 42, 34, 39], while other suggest that the same is happening while the alternative prey is abundant [22].

The conventional cluster analysis derived from radio-telemetry studies are the most common method for analysing Eurasian lynx diet [23]. Smaller prey, however, is much more difficult to encounter, because the procedure for discovering prey items is slower (the time between the kill is made, GPS/GSM locations are received and team visits the potential site) and therefore remains from smaller prey animals are almost impossible to detect. The classical VHF telemetry using homing-in methods to track lynx movements made it possible to encounter certain smaller prey items [17] that could otherwise be omitted. Another method to detect small prey is by snow tracking, but that would require good snow conditions that are not always the case. Nevertheless, the full prey spectrum could be concluded with scat analysis [35].

The Balkan lynx (*Lynx lynx balcanicus*, Buresch, 1941) presents an isolated and autochthonous population of the Eurasian lynx (*Lynx lynx* Linnaeus 1758) that inhabits south-western Balkans. The narrow distribution and limited number of mature individuals, as established by the expert assessment of its combined population at less than 50 adult individuals, earn the Balkan's largest felid the critically endangered (CR) status on the IUCN Red List of Threatened Species [27]. Hence, the lynx is strictly protected in the countries with confirmed distribution (Macedonia, Albania, Kosovo) and assumed distribution (Montenegro and Greece) [12]. The Balkan lynx diet hasn't been thoroughly studied in the past. Mirić's [29] monography on the Balkan lynx contains one chapter dedicated to diet in which anecdotal information on lynx prey spectrum are published, mostly coming from local hunters and herders. The project "Status, ecology and land tenure system of the critically endangered Balkan lynx *Lynx lynx martinoi* in Macedonia and Albania" [7] through the long-term and ongoing Balkan Lynx Recovery Programme enabled systematic radio-telemetry studies of the Balkan lynx. However, no activities have been dedicated to explore the smaller prey items that can be found in the Balkan lynx diet. Recent analysis conducted on Balkan lynx scats using basic forensic techniques like immunological analysis and identification of species through hair samples [11, 46, 18] suggests that the lynx feeds on a range of animals which pose an alternative prey, albeit some findings, as literature suggests regarding red fox, may hint to intraguild predation [42]. Documenting the alternative prey of an elusive predator such as the Balkan lynx is hard to determine, mostly because of the ecology of this medium-sized cat. Smaller prey is consumed immediately, often without a trace or evidence at the kill site. Additionally, in a habitat with scavenging animals, there are no leftovers to be traced and identified. The aforementioned forensic methods based on the scat analysis might help to discern what is on the feeding menu of the Balkan lynx.

We believe that this study will help shed more light on the feeding habits of this predator with hopes of improving conservation efforts. Thus, the aim of this paper is to get knowledge about Balkan lynx alternative prey represented in its diet.

2 STUDY AREA

Balkan lynx distribution is restricted to south-western Balkans, with two main nuclei with confirmed reproduction: Mavrovo National Park and its vicinity, in Macedonia, and Munella Mountain, in Albania [28]. The research presented in the study was performed in the Mavrovo National Park and its southern and eastern surroundings (Fig. 1). The area is typically mountainous with small rivers, steep valleys and an altitudinal range of 600 to 2,200 meters. The vegetation cover is mostly forests, represented by European beech (*Fagus sylvatica*), King Boris fir (*Abies borisii-regis*), various oak species (*Quercus* spp.) and mixed beech-fir forest communities. Besides lynx, two other large carnivores share the landscapes: brown bear (*Ursus arctos*) and grey wolf (*Canis lupus*). Wild ungulates present in the area which are potential food source for the lynx are the roe deer and chamois. The area is sparsely populated, where most of the people have abandoned the high-altitude villages and have settled into the nearby towns or migrated abroad [52].

3 METHODS

During a conducted radio-telemetry studies (2010–2018), obtained samples were mostly recovered near the feeding site (covered with litter, dry grass or snow), on trails and in box-traps (set to capture lynx). We used molecular biology technique similar to the DNA-based technique of species identification, with the advantage of low cost and potential to be performed in the field [53, 54, 55, 56, 57, 58, 59, 60]. DNA-based identification is more reliable and quite accurate, but it requires intensive laboratory work and it is more expensive.

Scat material was collected in the period between 2010 and 2018. Part of the scat was collected opportunistically during fieldwork (Balkan Lynx Field Book), while other is related to the collared Balkan lynx individuals (Fig. 1). In total, five individuals (4 males and 1 female) were captured and fitted with radio-collars. Lynx scats are particularly hard to find, as lynx tend to hide their excrements by covering them with nearby material (soil, dead leaves, snow, etc.). Searching effort to find kills and scats was calculated depending on the number of participants in the search party. Each participant would search the area in the centre of the cluster for one hour. The time was half an hour shorter if more than one participant joined the search party. When a kill was found, the search continued around the kill looking for a peculiar stack where scat is hidden. The scat was collected either in zipper bags or tubes filled with 70% ethanol. The samples were labelled with the following information: date, place, coordinates, lynx ID and habitat. Samples were then brought to the Forensic Department within the Macedonian Ministry of Interior (MoI) for detailed content analysis, including: morphological characteristics, determination of undigested parts of the prey item (hairs and bones), and immunological and serological analysis of the immunoglobulins found in the sample.

With the morphological analysis of the scat we could identify the species that produced the scat, i.e. whether it comes from a carnivore or a herbivore. Moreover, if the original morphology is preserved, the genus is identified as well. As regards detection of carnivores and brown hare in the sample, we proceeded with separation of the hairs from the rest of the sample content. Following physical separation, hairs were submerged in a physiological solvent for further cleaning. The final stage was rinsing the hair material in Xylene® (106.17 g/mol, Alkaloid). From the selection of treated hair samples, slides for oil immersion microscopy were produced using Canada balsam, dried on room temperature for 24 hours. Determination of origin of hair samples was made by comparison with hair material obtained with previously identified museum specimens from the mammal collection of the Macedonian Museum of Natural History in Skopje.

Possible protein residues from the faecal samples were diluted with 5% ammonium hydroxide solution (Sigma Aldrich®). This solvent was proved as most effective for extraction of old and denatured samples and that does not interfere with further testing [61, 62, 63]. Small piece of the samples, approximately 0.5 cm, was placed in 25 ml polypropylene screw cap tubes, together with 4.0 ml from the 5% ammonium hydroxide solution. During the first phase of dilution, the samples were placed on orbital mixer for a period of 1−3 hours, while later for the final dilution, sample tubes were transferred into the water bath with ultra sound in the duration of 2−3 minutes. The diluted sample was then transferred to a Petri dish and put in the airflow laminar until full evaporation. The evaporation leftover was than diluted in 200 µl sterile phosphate-buffered saline® (PBS, Sigma). Detection of residual proteins was performed in agar gel with pH 8.6. Wells with a diameter of 0.5 cm were arranged in a circular way into the agar gel plates, whereas the well in the centre was intended for the unknown test sample, and the wells for the specific antiserum were arranged in four directions goat and/or rabbit against bear, bovine, cat, hen, turkey, deer, dog, fox, human, rabbit, rat, mouse, sheep, horse and donkey. All antiserums were obtained in lyophilised form from MP Biomedical. The samples (unknown solvent) and specific antiserum were applied using Pasteur pipette into the designated wells and properly marked. Afterwards the .

at a distance of 0.5–0.7 cm. Used antisera are whole serum polyclonal unconjugated primary antibodies developed in agar gel plates with the samples were placed in a moist chamber where the immunodiffusion took place. The solvents diffused radially in the plate, and wherever there was a precipitation line on the touching surface between the tested sample and appropriate antiserum the analysis was considered positive.

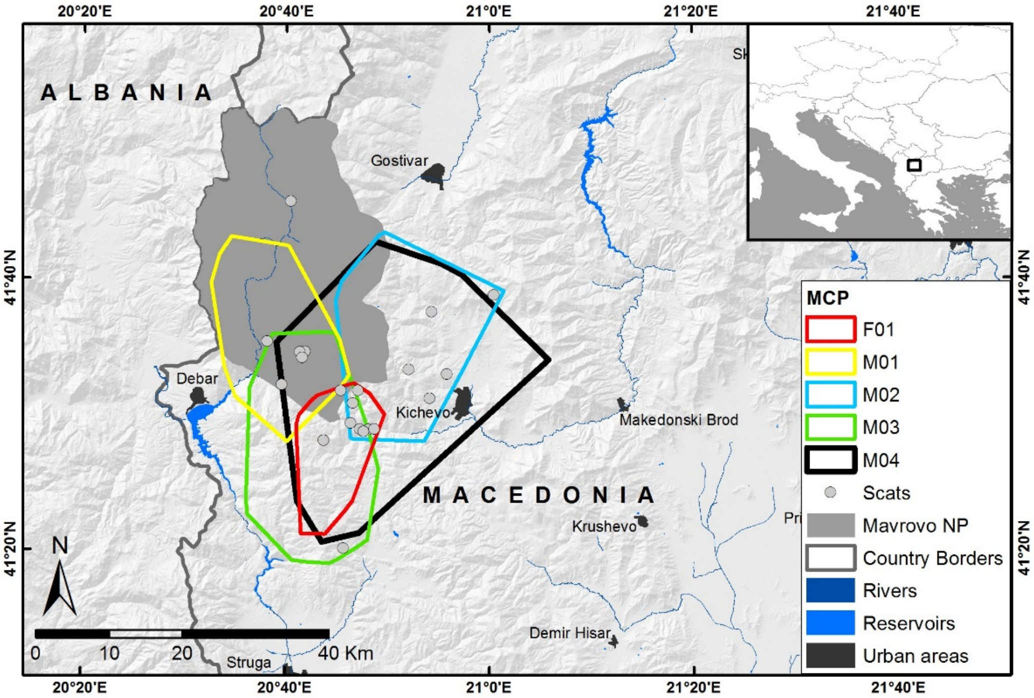

Figure 1: Study area. Grey dots are locations where scat samples were found. Rectangle polygons represent the home range of collared Balkan lynx

4 RESULTS

Until July 2018, a total of 149 field visits for finding prey on GPS clusters were performed. During these visits, 106 prey remains were found. Around the kills we were able to locate 24 scats. Furthermore, 13 lynx scats were found by chance. These scats could, in fact, belong to some of the radio-collared individuals. However, our analysis cannot confirm this. Of the total of 37 scats brought to the MoI laboratory, only 28 gave a positive result (Tab. I, Fig. 1). In total, 12 different prey animal species were detected in the scat remains: roe deer, chamois, brown hare, marten (*Martes* sp.), wild boar, rat (*Rattus* sp.), mouse (*Apodemus* sp.), edible dormouse (*Glis glis*), red squirrel (*Sciurus vulgaris*), shrews (*Neomys* sp., *Crocidura* sp.) and pipistrelle bat (*Pipistrellus* sp.).

The comparison of lynx diet among samples collected at kill sites (radio-tracking) and those collected randomly (by chance) revealed different results. For example, roe deer appears twice more (66.7%) in scat found at kill sites than in scat found by chance (33.3%). Furthermore, some prey species, like marten, edible dormouse, red squirrel, shrews and pipistrelle bat, are only found in scat collected at kill sites, while other (chamois, wild boar, rat and mouse) only in scat collected by chance. When it comes to prey detection per sample (scat), with radio-telemetry we could detect 1.3 prey sp. per sample compared to 1.2 sp. per sample with the scat found by chance, suggesting no significant difference. However, we should note that roe deer is the most present prey species in the scat (50%) collected. The previous relates to the fact that most scat is find at roe deer kills, which correlates to roe deer having the biggest share in the Balkan lynx diet (approximately 65%).

Concerning the lab methodology for prey detection in scat, hair analysis gave better results (39 prey items) in detecting prey species compared to the protein analysis (12 prey items). Additionally, hair analysis detected prey species in all 28 scats (100%), while protein analysis, in 13 (40%). This is due to the fact that proteins are more prone to degradation beyond detectability than hairs (Janevski. R. *personal communication*).

We did additional calculations to discover the frequency of occurrence (F%) showing the percentage of scats containing different food types relative to the total number of analysed samples; and level of significance (Fr%) – the frequency of occurrence in relation to the total occurrence of the food type. Results from that analysis is presented in Tab. II.

Table I: Results from the scat analysis of the Balkan lynx

No.	Date	Region	Animals confirmed		Scat retrieval
			Residual proteins	Hairs found	
1	No data	Mavrovo NP	Lynx*, chamois, rat, roe deer	Chamois, rat, roe deer	By chance
2	10.04.2010	Mavrovo NP	Lynx, roe deer, Brown hare, chamois	Brown hare, chamois	By chance
3	10.04.2010	Mavrovo NP	Lynx, roe deer	Roe deer	By chance
4	10.04.2010	Mavrovo NP	Lynx	Wild boar	By chance
5	24.02.2012	Kichevo region	Lynx	Roe deer	Radio-tracking
6	25.02.2012	Mavrovo NP	Lynx	Roe deer, brown hare	By chance
7	25.02.2012	Mavrovo NP	Lynx	Roe deer, mouse	By chance
8	07.03.2012	Kichevo region	Lynx, roe deer	Lynx, roe deer	Radio-tracking
9	14.03.2012	Kichevo region	Lynx	Lynx, roe deer, brown hare	Radio-tracking
10	11.04.2012	Kichevo region	No data	Roe deer	Radio-tracking
11	14.09.2012	Kichevo region	No data	Stone marten	Radio-tracking
12	27.09.2012	Mavrovo NP	No data	Brown hare	By chance
13	10.10.2012	Kichevo region	No data	Brown hare, roe deer	Radio-tracking
14	11.10.2012	Kichevo region	No data	Roe deer, marten	Radio-tracking
15	24.04.2015	Kichevo region	No data	Mouse	By chance
16	27.05.2015	Ohrid region	No data	Roe deer	Radio-tracking
17	19.03.2015	Kichevo region	No data	Lynx	By chance
18	15.03.2015	Mavrovo NP	No data	Roe deer	By chance
19	27.02.2015	Kichevo region	No data	Roe deer	By chance
20	24.01.2015	No data	No data	Roe deer, wild boar	By chance
21	10.04.2016	Kichevo region	No data	Edible dormouse	Radio-tracking
22	10.04.2016	Kichevo region	No data	Edible dormouse, pipistrelle bat	Radio-tracking
23	28.04.2016	Kichevo region	Brown hare	Brown hare, *Sciurus* sp.	No data
24	28.04.2017	Kichevo region	Brown hare	Brown hare, *Sciurus* sp.	Radio-tracking
25	23.11.2017	Kichevo region	No data	Roe deer, *Crocidura* sp., *Neomys* sp.	Radio-tracking
26	09.02.2018	Kichevo region	No data	Roe deer	Radio-tracking
27	01.05.2018	Mavrovo NP	Lynx, roe deer	Lynx, roe dear	Radio-tracking
28	No data	No data	Lynx, roe deer	Lynx, roe deer	Radio-tracking

*Lynx hairs and residual proteins belong to the same individual from which the scat was retrieved

Table II: Frequency of occurrence and level of significance results of different food type found in the collected Balkan lynx scat

Species	Frequency of occurrence (F%)	Level of significance (Fr%)
Roe deer	64%	44%
Chamois	7%	5%
Brown hare	25%	17%
Marten	7%	5%
Wild boar	7%	5%
Rat	4%	2%
Mouse	7%	5%
Edible dormouse	7%	5%
Red squirrel	7%	5%
Shrews	7%	5%
Pipistrelle bat	4%	2%
Total		**100%**

5 DISCUSSION

The chosen method of double immune diffusion on agar gel is a quick and cheap method for identification of antibodies such as immune globulins [64]. The method is called "double" for the fact that in this procedure, the antigen and the antibody are allowed to migrate towards each other in a gel and a line of precipitation is formed where the two reactants meet. The positive side of the precipitation reaction is its high specificity. The method is widespread and used regularly when working on diagnosis or protein detection, or when comparing antigens or antisera. The negative side of the method is its low sensitivity [1]. The considerable potential for prey identification was key to choosing this method although we targeted proteins of the most probable prey items known from previous ecological studies. While insufficient material was rare, negative results were obtained from samples that were more degraded or predominantly consistent of hairs. For these samples, adding the microscopic identification of hair significantly raises the chance of prey species identification. The macro- and microscopic structure of mammal hair is amazingly diverse and therefore easily discernible for species, genera or higher taxa. Mammal identification through hair morphology requires time and practice, and so this method is underproportioned in the era of molecular investigations. At the same time, hair is the most frequent and often the only life sign of mammals in the field. Moreover, the most recent biodiversity and conservation biology studies prefer the use of non-invasive methods, including hair-trapping, bird-nest analysis, and the identification of the hairs as remnants of prey taxa [43]. We recommend using both methods (hair and protein analysis) because they provide best results when combined, although hair analysis seems to be the more reliable method.

In view of the results presented above, roe deer is evidently the principal prey of the Balkan lynx, with a significant diet contribution found in 18 scats. This is in line with diet studies conducted in other regions of the nominal taxon's distribution, namely, the Norwegian [34], Swiss [8] and Dinaric populations [22]. In the Balkans however, the negative anthropogenic pressure on roe deer population within the home range of the Balkan lynx manifests in various forms: logging (either legal or illegal), hunting, poaching, and presence of stray dogs. Such occurrences, in turn, cause population decline and distress in the prey species consequently reducing the lynx's prey availability. Therefore, although larger than its Spanish and North American counterparts, the Balkan lynx might alternatively feed on small mammals. In that respect, lagomorphs become main prey whenever roe deer availability is reduced, and in our study they were found in seven analysed scats. Thus, main prey lows make the brown hare a significant food source for the lynx, especially in exploited forests [15]. Moreover, prey selection in lynx, as already pointed out, also hinges on age and sex. Brown hare seems to be the main prey species of subadult individuals, whereas adult males tend to hunt larger preys than females [38, 35]. Chamois also participates into the lynx prey base (twice found in the scats), albeit to a lesser degree than the previous two prey items. This may be due to the restricted distribution range of this bovid and its modest population size [2]. The additional prey data reflects the opportunistic predatory nature of the Balkan lynx. This is seen in the contribution of marten into its prey base (found in two scats). Lynx predation upon wild boar is low, whereby our scat analysis shows that suids are found in two scat samples. This may suggest either predation upon juvenile individuals or scavenging – the latter is rarely seen in lynx [3, 36, 41, 34, 5, 22]. Furthermore, scarcity of the main and the preferred alternative prey provokes the lynx to hunt shrews and rodents [15, 45, 34, 22], despite their low nutritional value. Our results confirm this and reveal that the Balkan lynx predates upon mouse (found in 2 scats), shrew (2), squirrel (2) rat (1), and dormouse (2). In fact, edible dormouse presents an important alternative prey for the Dinaric population of Eurasian lynx, particularly for females and subadults [22]. An increase in the frequency of finding hares and rodents may be seasonal and more research is needed to have a clear picture. As far as opportunism goes, one peculiarity of behaviour the Balkan lynx displays is predation of bats. Scat analysis confirms the presence of pipistrelle bat (found in one scat analysis) in the lynx diet. This novel finding is the first ever to indicate such behaviour in the Balkan lynx, although some literature suggests that bats can become occasional lynx prey [14] (Jędrezejewska & Jędrezejewski 1998). Since rats and, to a great extent, pipistrelle bats are readily associated with human habitation (mostly low-activity or derelict buildings), the finding implies that lynx might approach man-made infrastructure. This is further supported by the fact that the Eurasian lynx uses conspicuous human objects in its natural surroundings for scent-marking [40, 21]. But ventures into human settlements are sometimes prey-motivated, as seen in the attacks on livestock, which are mainly due to the reduced availability of roe deer [34]. Still, despite the anecdotal evidence of such behaviour in our study area, scat analysis reveals no traces of livestock animals. However, another point to consider in this respect is scat sample size. Trites and Joy [44], for example, recommend that a minimum of 59 scats should be analysed to identify prey species occurring in scat at a frequency below 5%. Contrary to numerous bird findings in Eurasian lynx diet [16, 38, 42, 45, 34, 22], our investigation does not show presence of this prey item. This may be owing to the relatively small sample size (28 scats that gave results). In that respect, Pires *et al*. [37] warn against underestimating avian prey when poorly represented in scat, especially if the scat contains mammalian prey vestiges.

6 CONCLUSIONS

The paper makes an initial attempt to elucidate the full prey spectrum of the Balkan lynx diet. The variety and the frequency of consumption of alternative prey can be considered as an indicator of the principal prey availability in disturbed habitats. The methods we used for scat analysis can help detect the smaller alternative prey in the Balkan lynx diet that is impossible to discover with the field search using lynx telemetry locations. Described methods (immunological and hair sample analysis) are reliable and precise, they are cheaper than DNA analysis, but still require laboratory work. Future application of these methods will be crucial to establish their relevance in such investigation and validate results gained. Having information on the whole lynx prey spectrum can help in understanding the predator-prey relationship, competition and prey availability which then can be used for sustainable management of main prey species as an important precondition for the survival and recovery of the Balkan lynx.

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