Published by Associazione Teriologica Italiana
Hystrix, the Italian Journal of Mammalogy
Volume 29 (2): 190–194, 2018
OPEN ACCESS
Available online at:
http://www.italian-journal-of-mammalogy.it
doi:10.4404/hystrix-00078-2018

Research Article

A new sampling method to detect the Pyrenean desman (*Galemys pyrenaicus*)

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Keywords:
detection
sampling method
desman
metabarcoding
scats.

Article history:
Received: 6 March 2018
Accepted: 8 November 2018

Acknowledgements
This work was funded by the EU project LIFE IREKIBAI (LIFE14 NAT/ES/000186) and the Basque Government. Amaiur Esnaola enjoyed a predoctoral grant by the Basque Government. Authors are very thankful for Arturo Elosegi and Aitor Arrizabalaga for their assistance in making this work possible, and also for technical and human support provided by Secuencing and Genotyping Unit – Genomic Facilities – SGIker (UPV/EHU/ERDF, EU).

Abstract

The Pyrenean desman (*Galemys pyrenaicus*) is a small semi-aquatic mammal whose populations have suffered a severe decline in recent decades. Its conservation requires a monitoring program to quantify changes in their populations and distribution. Scat surveys have usually been carried out for this purpose, but they tend to yield a low success rate, which depends on local environmental conditions such as channel form and lithology. This methodological constraint causes that part of the population goes unnoticed. In this study a new method for the detection of this elusive species is tested. The research has been done in the Basque Country (Northern Iberian Peninsula), in Elama and Leitzaran streams, where desmans have been recently recorded. Artificial shelters have been placed, regularly distributed in both streams, offering desmans suitable places to rest and defecate while foraging. The desmans used quickly and repeatedly the artificial shelters, significantly increasing their detection rate. The field identification of scats, based on their shape, colour, size and odour, was subsequently confirmed by DNA analyses with metabarcoding. This new non-invasive method allows obtaining fresh faecal samples of known age, making them available for further studies on genomics, population genetics, dietary studies, reproductive analyses, etc. The low cost of the materials used and the possibility of identifying desman scats after basic training, make this method optimal for synchronic, regional-scale and/or volunteer-based surveys. Thus, the use of artificial shelters results in a substantial improvement over traditional desman scat surveys, and greatly enhance the means for future monitoring of the populations of this endangered species.

Introduction

Conservation of endangered species requires to periodically determining their distribution area as well as demographic parameters of their populations. Because of practical constraints, periodic follow-up is usually possible only if there are accurate and relatively cheap detection methods that are easy to implement. The elusive character and nocturnal habits of most mammals forces researchers to mainly rely on their tracks and signs for surveys (Wilson et al., 1996). The tracks of some species are unmistakable, what allows defining survey protocols based on the search of traces (e.g., Helle et al., 2016), although in environments where tracks are hard to detect faeces can offer a suitable alternative. Faeces can be useful to monitor a species if they can be unequivocally assigned to that species and if the animal deposits them in a predictable way in places accessible to the researcher. Nowadays techniques in molecular genetics allow correct identification of the species that produced a scat (e.g., Janecka et al., 2008; Ruiz-González et al., 2008; Gillet et al., 2015; Walker et al., 2016), although these methods require expensive procedures and specialized technicians, thus making it difficult to intensively survey large geographic areas. Direct identification by observation is a much-preferred option, when available. A good example is the survey of Eurasian otter (*Lutra lutra*), which is periodically performed in Europe (Mason, 1986). These surveys, generally performed at a regional scale, involve a large number of volunteers who are previously trained to acquire the necessary skills. The distinct characteristics of its scats (form, smell, colour, specific layout on the ground) make it possible to get enough reliable observations as to outline the distribution of the otter in a wide area (in the order of thousands of km^2) in a short time frame.

The Pyrenean desman (*Galemys pyrenaicus*) is a semi-aquatic mammal of 60–70 g. It usually seeks shelter in natural crevices between rocks, in stone walls, or below the roots of riparian trees; usually there are no outwardly visible signs of the shelters from the surface (Stone, 1987). It feeds mainly on freshwater invertebrates (Santamarina and Guitián, 1988; Biffi et al., 2017; Hawlitschek et al., 2018) and, as the otter, lays its faeces on emerging structures in the stream channels (mainly rocks, logs and roots). Desman scats can unequivocally be assigned to the species when fresh, by their smell, colour, shape and size (Nores, 1992), but when they get older their scent vanishes, their colour changes and if they get dry they disintegrate easily, what makes them more prone to misidentification with other small mammals such as *Neomys sp.* or *Rattus sp.*, among others (see Gillet et al., 2015). Despite these problems, scat surveys have been used to monitor desman populations through their distribution area (Nores, 1992; Bertrand, 1992; Queiroz et al., 1998). Sometimes these surveys yield a low success rate (low number of scats per surveyed reach) (González-Esteban et al., 2011; González-Esteban, 2014; Charbonnel et al., 2015), which also depends on channel form and lithology of the streams. In reaches with limited favourable substrata such as emerging rocks and logs, desmans defecate in burrows or in holes below overhanging banks, out of the reach of the surveyors (González-Esteban et al., 2003). Therefore, some populations are not detected in routine surveys. There has been some discussion on how to correct these false absences, but the problem still remains unsolved (Charbonnel et al., 2014).

The Pyrenean desman is protected under the Bern Convention (Appendix II) and the EU Conservation of natural habitats and of wild fauna and flora Council Directive (Annexes II and IV) (Council of the

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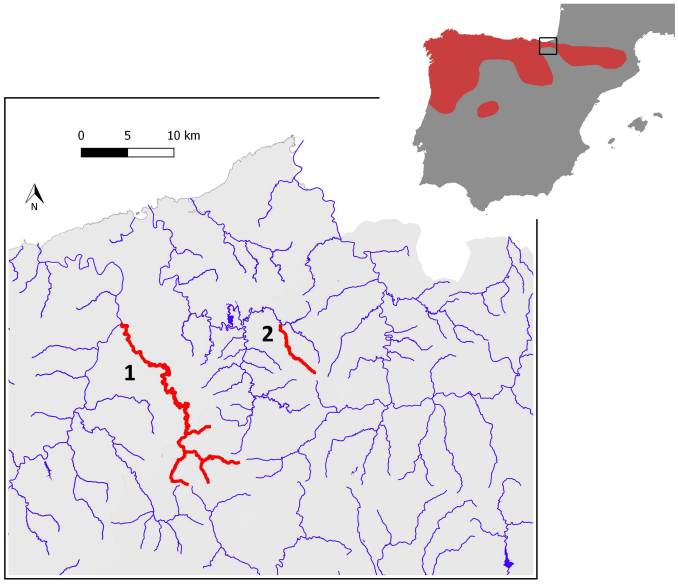

Figure 1 – Study sites: (1) Leitzaran and (2) Elama Streams, in the northern Iberian Peninsula.

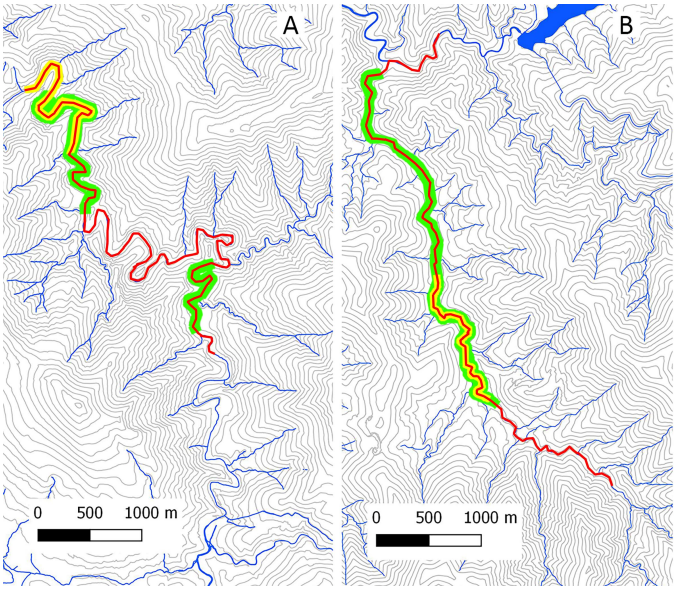

Figure 2 – Study sites, in more detail: (A) Leitzaran and (B) Elama Streams. Information about the sections analysed in the active search for desman scats (red line), about the sections analysed in the first trial of the artificial shelters (yellow line) and about the sections analysed in the second trial of the artificial shelters (green line) was added in the figure.

European Communities, 1992). Its distribution area has been severely reduced during the last decades (Charbonnel et al., 2016; Quaglietta et al., 2018) and it is currently listed as a vulnerable species in the Red List Categories by the IUCN (Fernandes et al., 2008). The problems above outlined about detection of signs of presence — namely the varying success rate, the high cost and the risk of false negatives — prevented detailed large-scale-surveys of the desman. Consequently, its current distribution area is only partially known and no global survey has hitherto been conducted. This could become extremely serious since at least five genetically discrete conservation units have been identified for this species (Querejeta et al., 2016), which should have specific management approaches. Here we show a new detection method that can help solving the main limitations of the current surveys, making desman detection faster, cheaper, and reducing the risk of false absences.

Materials and methods

This study was carried out as part of a broader research on the spatial and trophic ecology of the Pyrenean desman conducted in two mountain streams in the northern Iberian Peninsula: Elama Stream (330 m mean altitude; 7 m mean width; 0.7 m^3/s mean flow) and Leitzaran Stream (290 m mean altitude; 12 m mean width; 4.6 m^3/s mean flow) (Fig. 1). Both are protected under the EU Natura 2000 network, both are in good ecological status according to the EU Water Framework Directive (Council of the European Communities, 2000), and the presence of Pyrenean desmans has been known for at least 30 years in both (Nores, 1992; Esnaola et al., 2018).

These streams show contrasting environmental pressures. Elama is a second-order headwater stream draining an uninhabited basin of 1415 ha over granite and schist that has been managed strictly as a nature reserve since 1919, resulting in extensive cover of beech and oak forests (Castro, 2009). At present there is no extractive activity in the Elama basin. On the other hand, Leitzaran is a fourth-order stream draining a basin of 12402 ha over limestone, slate, and sandstones. Unlike in Elama, in the headwaters of Leitzaran there are two towns totalling 3150 inhabitants, after which the stream enters an uninhabited valley, approximately 25 km in length, where forestry and hydropower diversion schemes are the main human activities (Izagirre et al., 2013). The characteristics of the channel are different in both streams: in Elama riffles and runs are similarly available (45%), whereas runs are dominant in Leitzaran (60%). Pools are the least abundant habitat in both streams (10%). Both streams have emerging blocks and logs in the riverbed and in the riverbank. These elements are more abundant in Leitzaran, providing more depositional zones (places to deposit their excrements) to the inhabiting desmans. The vegetation cover is scarce in both streams, mostly herbaceous and shrubby, being easily accessible to the observer.

In a preliminary sampling trial, in March 2016 we searched for desman scats in a 6 km segment of Elama Stream and an 8 km segment of Leitzaran Stream, following the standard active search procedure defined by Queiroz et al. (1998). In a situation of base flow and after 10 days without rain, we surveyed the streams inspecting with a hand torch all structures in the channel and on the banks where desmans could defecate (mainly rocks and logs). Each stream segment was examined in its entire length, without differentiating sections, for three consecutive days by 3 people, at an average standard speed of 200–300 m/h. During the surveys, the water level of both streams showed no fluctuations.

Next, we tested the efficiency of attracting desmans to home made artificial shelters or latrines. These artificial shelters were built with high-density ethylene-vinyl acetate (EVA) foam mats (ref. B517V1, mottez.com, 6 € each, 630×630×12 mm). Each mat was bent on top of a mound of boulders built in the middle of a shallow (10–30 cm deep) riffle, forming a "tent" that covered a dry platform over 0.25 ^{2}m. The mat was fixed in place by means of rocks, piled as to offer a flat surface that could be easily accessed by desmans and would guarantee the persistence of droppings (Fig. 3, 4), and it was tied by a string to a nearby branch to prevent losing it in the event of a flood. The space between the rocks and the roof of the "tent" was not larger than 15 cm. The resulting structure thus simulated a small shelter or latrine, similar to those used by desmans to rest while they eat the food captured underwater (Niethammer, 1970). The structure was located in riffles, as these are the best places for desmans to capture food (Richard, 1986; Esnaola et al., 2018).

To test their efficiency, we carried out a first sampling trial with artificial shelters in two smaller stream sub-sections of the same river stretches (1.8 km in Leitzaran and 1.4 km in Elama), where we did not find desman scats during the initial active search. We sampled the subsection of Leitzaran in April-June 2016, immediately after the first standard active search trial carried out in March, whilst the sampling in Elama was delayed until June 2016. We set 12 artificial shelters in Leitzaran Stream and 18 shelters in Elama (Fig. 2), their numbers and locations depending on the availability of riffles, with a mean distance of 160.9 m between them in Leitzaran (SD: 60.2 m, range 55–210 m) and 82.9 m in Elama (SD: 22.9 m, range: 55–135 m). The shelters in Leitzaran were kept for 92 days and checked every 10–15 days, whereas

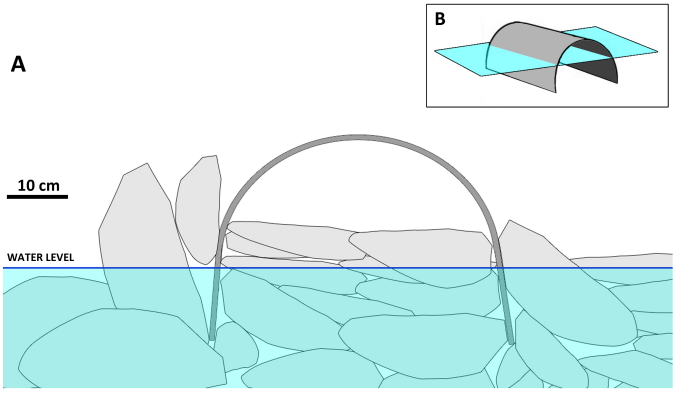

Figure 3 – Scheme of a shelter.

those in Elama were kept for 13 days and surveyed each 6–7 days. It is worth mentioning that sampling in Elama was suspended after 13 days because all the shelters got collapsed with scats. In both cases the streams registered base flow and little or no rainfall during the sampling time. On each visit we collected all the droppings found and preserved in individual vials in absolute ethanol, and all shelters were cleaned up to avoid finding the same scats later.

Later on, taking advantage of a subsequent trapping and radiotracking study, developed in September-October 2016 (Esnaola et al., 2018), which provided precise information of the stretches occupied by the desman in both stream stretches, we carried out a new trial to better assess the effectiveness of sampling with artificial shelters. Thus, in October 2016, also with base flow and low rainfall, we set 16 shelters in each of the streams, in reaches where desman presence was confirmed by trapping and radio-tracking (Esnaola et al., 2018). This time we sampled a 3.5 km long section (two subsections of 1.0 km and 2.5 km) in Leitzaran, and a 4.0 km long section in Elama, and the shelters were again distributed on shallow riffles (Fig. 2). The mean distance between shelters was 233.3 m in Leitzaran (SD: 47.3 m; range 190–360 m) and 270.3 m in Elama (SD: 67.3 m; range: 170–410 m). All shelters were surveyed every 24 h for three consecutive days and all scats were collected as described above. To minimise the loss of scats due to possible water level fluctuations, the shelters were surveyed early in the morning, just after the end of the desman nocturnal activity period. After each visit, all the shelters were cleaned up to be sure that scats found in a shelter were new at next visit.

The faeces were screened in the field, and only those with characteristics of desmans (musky smell, long, braided shape formed by the fusion of small spheres, length 15–30 mm) were preserved in individual vials in absolute ethanol.

Species identification by DNA metabarcoding

The source of the scats was identified by DNA metabarcoding of 15 random faeces collected in the first trial and almost all the faeces collected in the second one (N=150), as part of a molecular study of desman's diet (unpublished). Mitochondrial Cytochrome c oxidase subunit I (COI) gene was extracted using the Qiagen Powerfecal DNA kit (Qiagen Iberia, S.L. Madrid), following the manufacturer guidelines. Subsequently, DNA was PCR amplified from extracts at the Analytical Services (SGIker) of the University of The Basque Country UPV/EHU, using the primer set described by Gillet et al. (2015). Samples were purified and a second reaction was performed to index each amplified product and attach Illumina adaptors using the Illumina Nextera v2 kit. Amplifications were performed with the Quiagen Multiplex PCR Kit protocol, using 12.5 μL Quiagen 2$\times$ (1$\times$ final), 1.25 µL forward primer (10 μM; 0.5 μM final), 1.25 µL reverse primer (10 μM; 0.5 μM final), 8 μL H2O and 2 μL DNA, in a final volume of 25 μL, with one activation step at 95 °C for 15 min followed by 40 cycles (denaturation at 94 °C for 30 s, annealing at 45 °C for 45 s, extension at 72 °C for 30 s) and final extension step at 72 °C for 10 min.

Once amplified, sequencing of PCR outputs was carried out in an Illumina MiSeq NGS platform (sequencing of 2x300 bp paired-end reads) with the MiSeq©Reagent Kit v3 (600 cycle), following the manufacturer instructions. Sequencing was performed at the Analytical Services (SGIker) of the University of The Basque Country UPV/EHU.

Paired-end reads were merged using USEARCH (Edgar, 2010, 2013; Edgar and Flyvbjerg, 2015), demultiplexed by primers, adapter and primer sequences were removed, and reads were quality and length filtered using CUTADAPT (Martin, 2011). Then, singletons were removed and the remaining sequences were screened for chimeras using USEARCH. UPARSE algorithm (Edgar, 2013) was used to cluster sequences into Operational Taxonomic Units (OTUs) at a 97% similarity threshold (see Alberdi et al., 2017). Finally, Genbank nt database was used to assign taxonomy to OTUs using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Species level assignments were performed when sequences matched with 100% similarity and 100% overlap, following Clare et al. (2013).

Results

The preliminary sampling based on the active search standard protocol (Queiroz et al., 1998) yielded no desman faeces in the Elama Stream, and only two scats in the Leitzaran Stream (0.25 faeces/km). The desman shelters yielded a much higher number of faeces. On the first trial with artificial shelters (April-June) in sections with no previous confirmation of desman presence, over 50% of the shelters yielded faeces, and their production sustained over time (Tab. 1A). The deposition rate was higher for Elama than for Leitzaran Stream (24.5 vs 11.1 scats per km and survey, respectively). Actually, on the first trial, the visits to check the shelters were suspended in Elama after 13 days due to the intensive use of them by desmans. Visiting the shelters on a weekly basis, the stack of scats impeded individualizing samples. That situation was not observed in the Leitzaran.

On the second trial with artificial shelters (October) in reaches where desman presence was confirmed beforehand, over 80% of the shelters were used by the 3rd night (Tab. 1B). As in the first trial, the deposition rate was higher for the Elama than for the Leitzaran Stream (10.6 vs 4.5 scats per km and survey, respectively).

In spring samples around 12667575 raw DNA sequences were obtained from faeces, which were reduced to about 11788479 after quality filtering. In autumn, instead, around 10297382 raw DNA sequences and about 8724646 filtered sequences were got. Bioinformatics' analyses and blasting showed that all the faecal samples contained abundant DNA sequences corresponding to the Pyrenean desman (100353 filtered reads per sample on average in spring and 10153 in autumn) with a 100% of similarity with the reference sequences. No sample

Figure 4 – Photograph of a shelter. Photo by Amaiur Esnaola.

Table 1 – Numbers of scats collected in the study streams with artificial shelters. For each survey, it is shown the time elapsed since the shelters were set up (T, days), the number of shelters visited by desmans (P) and the number of faeces collected (N).

A) First trial. Shelters in the Leitzaran Stream were set up on 13th April 2016, those in Elama Stream on 11th June 2016

	Leitzaran (12 shelters)			Elama (18 shelters)		
Survey	T	P	N	T	P	N
1	16	7	32	6	13	27
2	28	5	17	11	12	34
3	41	3	7	13	12	42
4	56	9	20			
5	68	4	15			
6	81	10	32			
7	92	8	17			
	Total	10	140		13	103

B) Second trial. Sampling with surveys every 24 hours. The shelters in the Elama Stream were set up on 1st October 2016, those in the Leitzaran Stream on 30th October 2016

	Leitzaran (16 shelters)			Elama (16 shelters)		
	T	P	N	T	P	N
	1	5	15	1	11	38
	2	6	15	2	14	42
	3	11	24	3	12	32
	Total	13	54		15	112

was excluded because of low read numbers or bad sequence quality. No other potential source of faeces was identified.

Discussion

Population density of the Pyrenean desman ranges from 4 to 8 ind/km (Nores et al., 1998), desman individuals prospecting daily their home ranges (Stone, 1987). Although the rate at which they defecate is unknown, it is not unreasonable to estimate that tenths of scats can be produced per day and km, what, even with a large rate of loss, would yield hundreds of scats per km available, at least during dry, base flow periods. Nevertheless, these numbers contrast with the low yield collected during surveys, which ranges from 1.7–1.8 to 5.8 scats/km (Charbonnel et al., 2015). The difficulty to find desmans' faeces in some streams occupied by the species produces false absences obtained through active search of scats (e.g. González-Esteban et al., 2003), which are a matter of concern for administrations that carry out regional and national inventories since the 1980s. Recent studies (Charbonnel et al., 2014) have worked to correct this problem.

The probability to detect desman faeces seems to be related to the composition and structure of stream channels (González-Esteban et al., 2003), being higher in channels with abundant emerging blocks accessible to the surveyor. The present study supports this hypothesis: the desman responds to an artificial increase in emergent structures, using them rapidly and continuously, and thus making it easier to detect it. Nevertheless, further research should be carried out to check the response of desmans to artificial latrines in streams with high availability of emergent structures or shelters.

On the other hand, it is difficult to explain the differences in yield of the artificial shelters among the studied streams, and our sampling design wasn't designed to do so. These might reflect either a higher population density in the Elama Stream (Esnaola et al., 2018), or simply a higher relative increase in the surface of emergent structures per unit of surface area as a consequence of the building of artificial structures, what would trigger a stronger response by the desmans. In fact, as local deposition rates may be conditioned by many factors, researchers should keep from using them to infer any quantitative conclusion.

Whatever the case, the new sampling method presented in this paper greatly increases the detectability of desmans, providing an improved tool to test the species presence/absence with less risk of getting false absences. Additionally, it makes it easier to define the age of the faeces, thus improving the efficiency of methods that need abundant fresh material, such as genomics, population genetics, landscape genetics, dietary studies, or reproductive analyses (sex hormones). In particular, the need to find a method that allows the collection of fresh desman faeces has been highlighted by several authors (Gillet et al., 2016; Hawlitschek et al., 2018).

Moreover, this method is based on cheap, easy-to-use materials; building the shelters can be easily learnt in a single session, and two people can build 4-6 shelters per hour. Besides, even though this sampling procedure will always require two visits to the study area — to set the shelters first and to check them later —, it is noteworthy that checking for faeces in fixed spots is much easier and less time-consuming than a full survey of all putative latrines along a stream stretch. Additionally, the high yield of fresh faeces favours in situ identification after basic training. Therefore, this method seems optimal for synchronic, regional-scale surveys, especially if they involve volunteers, as used for other mammals such as the water mole *Arvicola amphibius* in the UK (National Water Vole Monitoring Programme; https://ptes.org/get-involved/surveys/countryside-2/national-water-vole-monitoring-programme/).

Artificial shelters, thus, offer an important improvement over traditional desman scat surveys, and greatly enhance the possibility for future monitoring of the populations of this endangered species based on non-invasive sampling methods. Future work should define the protocol to use in these surveys (number of sampling units, effort, seasonality, etc.).

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Associate Editor: P. Colangelo