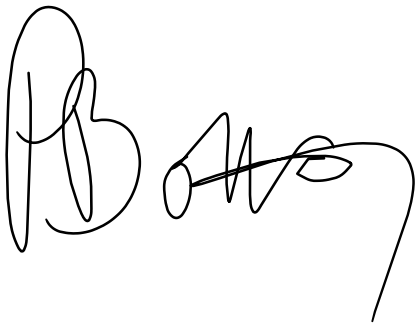
Comparing fine-scale temporal environmental DNA (eDNA) sampling to camera trapping for detecting mammalian carnivores

By Polly Louise Bolton

I hereby declare that the following dissertation is entirely my own work and has not been submitted, in whole or in part, for any award at this or any other academic institution.

SIGNED 

Contents

Abstract

Anthropogenic pressures have caused global species declines, making accurate biodiversity monitoring essential in providing data to help avert extinctions. One-fifth of mammalian species globally are classed as under threat which means a rapid, reliable, and inexpensive monitoring method could help streamline conservation management decisions by following patterns or species dips/increases in the data. As many monitoring techniques are invasive or destructive, a non-invasive technique would reduce direct handling and stress to the animals. Environmental DNA (eDNA) has emerged as a cost-effective, non-invasive, and efficient biodiversity monitoring method which has been used for both aquatic and terrestrial species. Previous mammalian eDNA-based studies have effectively detected terrestrial and semi-aquatic mammals and compared favourably to camera trap results. However, carnivores are one of the more problematic groups regarding eDNA detections. In this study, the validity of semi-aquatic and terrestrial mammalian detection via eDNA was investigated within the north-eastern region of Spain by comparing eDNA metabarcoding to camera trap data at 25 sites along five rivers. Two eDNA sampling events were carried out 10 days apart to see if short term temporal sampling would validate results. 24 species were detected overall from the eDNA samples from five different orders, 22 species were detected in the second sampling period whereas only 18 were detected in the first sampling period. Camera traps were set to run for 10 days between the first eDNA sampling session till the second.

Species richness was noticeably higher when combining the samples. Six species of carnivores were detected with eDNA and seven by the camera traps, with five species overlapping. All six carnivores were detected in the second sampling period with only four detected in the first. Detection probabilities (using occupancy modelling) were largely similar for carnivores when using eDNA and camera traps. The successful detection of multiple carnivores within this study has shown how temporal eDNA sampling can provide rich and robust presence-absence data which aligns with traditional methods.

1. Introduction

1.1 Mammalian species decline

With an estimated 28% of globally assessed species threatened with extinction, robust and reliable biodiversity monitoring methods are more crucial than ever (IUCN, 2021). There is an estimated range from 5475 to 6399 extant mammal species globally according to the IUCN and 2018 Mammal Diversity Database respectively (Burgin et al., 2018). Mammals are under threat from factors such as pollution (Temple and Terry, 2007), climate change (Maiorano et al., 2013), human population growth (Avila, Kaschner & Dormann, 2018), invasive species (Duenas et al., 2021), habitat loss and hunting (Brodie, Williams & Garner, 2021). Due to the increased mammalian extinction risk, there is an urgency to develop reliable and accurate non-invasive biomonitoring techniques that can contribute towards both creation and implementation of conservation policy (Sales et al., 2020a). Global concern regarding species decline has led to politically agreed international targets to reduce rapid loss such as the Convention on Biological Diversity's Aichi Targets (Convention on Biological Diversity, 2021). However, due to deficiencies in some nations' biological assessments and a consequent lack of legislative response, biodiversity declines are not being addressed appropriately (Collen et al., 2013). Long term monitoring of populations is crucial in detecting early signs of species decline and potential extinctions and should allow optimal time for conservation management practitioners optimal time for intervention (Lindenmayer et al., 2012). Funding is often a large hurdle for extensive monitoring systems, one solution is the use of citizen scientists who volunteer to collect data which would allow for a greater abundance of information collected (Biggs et al., 2015). However, appropriate measurements should be taken to ensure correct and standardised monitoring and reporting as the quality of the data needs to be analysed for trends and its potential to be used for decision making (Chandler et al., 2017; Lindenmayer et al., 2012).

1.2 Traditional monitoring methods for mammals

Monitoring methods we term as 'traditional' often focus on the detection of single or multiple similarly sized species (Wearn & Glover-Kapfer et al., 2019). Terrestrial mammals are traditionally monitored through camera traps (Cutler & Swann, 1999), track identification (Franklin et al., 2018), live traps (Blair, 1941), hair traps (Chiron et al., 2018) or scat identification (Birks et al., 2005). These methods are usually species or size specific so are unable to record data on whole communities simultaneously. The exception to this is camera traps which are successfully and widely used for biodiversity research due to their ability to record multiple species at once (Wearn & Glover-Kapfer et al., 2019). Live trapping is one of the more invasive monitoring methods which brings a level of risk to both researcher and animal during handling, especially in large and dangerous species (Sheils et al., 2018). Therefore, it is not always ideal for larger mammals but can be used for smaller mammals. However, mortality rates in small mammals are particularly elevated due to high metabolic rates leading to starvation, as seen commonly in shrews (Do, Shonfield and McAdam, 2013) or due to predation of the trapped animal (Evrard and Bacon, 1998). Animal handling may also require permits making it a potentially expensive and labour-intensive monitoring method which is often only 'single-catch', meaning it can only be utilised on select species (Sheils et al., 2018; Wearn and Glover-Kapfer et al., 2019). Live trapping allows for a more accurate species identification that may be missed by other methods, such as distinguishable species identifiers, and researchers can take samples, mark with dye or collard rings, or GPS radio-tag individuals for population-level research (Sheils et al., 2018; Wearn & Glover-Kapfer et al., 2019). It has been shown that behaviour can influence individuals who are captured, animals in poor body condition are more likely to enter traps for bait, and to achieve unbiased results it is best to use more than one live trapping method (Bisi et al., 2011).

One method of non-invasive biodiversity monitoring comes from field cues. Field cues can be animal tracks, hair traps or scat identification. Animal tracks being footprints or distinctive marks left by individual species, hair traps are sticky materials left in a study area to catch hair or fur from a passing

animal and scat identification is using faeces to identify a species, this method is commonly used for carnivore identification.

These methods come with a high degree of misidentification, even to specialists, which can have large impacts on the accuracy of data. Similarly, species may not be identified due to not being detected within the study area, however this problem will arise with most sampling techniques. Carnivore monitoring studies have reported difficulty with identifying scat (Grimm-Seyfarth et al., 2019) due to similarity in morphology and, in the case of the Eurasian otter (*Lutra lutra*) and American mink (*Neovison vison*) scat, similarity in diet. Human error rates in scat identification have been shown to vary from 14% (Red fox *Vulpes vulpes*) to 88% (wildcats *Felis silvestris*) when comparing human identification to DNA sequencing (Monterroso et al., 2013). Harrington et al. (2010) emphasises the usefulness of DNA sequencing for accurate species identification after false positives of presence of North American mink (*Neovison vison*) via scat were later shown to be left by other carnivores using DNA sequencing of mitochondrial DNA (mtDNA). Grimm-Seyfarth (2019) reported success in sniffer dogs differentiating between otter and mink scat at a 95% success rate in a laboratory setting. There has been some reported success in conservation dogs detecting scat from koalas (*Phascolarctos cinereus*; Cristescu et al., 2015), tigers (*Panthera tigris*; Kerley, 2010), black bears (*Ursus americanus*), fishers *(Martes pennanti*) and bobcats (*Lynx rufus*; Long et al., 2007). Although the results for using trained domestic dogs for species detections looks promising there is some reservations over training guidelines and appropriate canine selection due to the lack of literature (Beebe et al., 2016). Despite the early promise of detection dogs' further studies surrounding costs, surveying techniques, canine selection and training and detection type could further promote and help future researchers (Bennett, Hauser and Moore, 2020).

Hair traps are often one of the more favourable non-invasive monitoring techniques due to the low cost-effort ratio and availability of over-the-counter materials (Castro-Arellano et al., 2008). As DNA

can be extracted from hair, this can ensure correct identification can be collected from this non-invasive technique (Browett et al., 2020).

In the early 1900s the first "trap camera" was used to capture wildlife by George Shiras III, since then camera traps have firmly remained as one of the more popular biodiversity monitoring 'traditional' methods when compared to the others (Sanderson & Trolle, 2005; Wearn et al., 2019). Most traditional methods can be labour intensive in both set up and analysis, camera traps can present further problems such as malfunctions and unclear pictures for species identification, they have positive trade-offs such as the ability to provide a broad spectrum for biodiversity studies due to the 'multi-catch' ability, recording multiple species at once (Davis et al., 2018; Wearn et al., 2019). This allows researchers to build occupancy models based of presence and absence data, including the presence or absence of invasive species which would allow for management decisions (Davies et al., 2018). Camera trap images can provide researchers with a broad data set from the animals such as distinct markings, age, measurements, and group sizes. Camera traps have been successful in estimating abundance in tigers *Panthera tigris* where unique markings can differentiate individuals (Karanth, 1995). Camera trapping's simplistic methodology allow for standardised repeatability added to the low costs, making this method popular for capturing mammalian communities (Cove et al., 2021), although camera trap studies only capture portions of heterogenous landscapes and can often miss smaller species meaning alternative methods will have to be used to monitor small mammals (Harper et al., 2019; Leempoel et al., 2020). These traditional methods are widely used for terrestrial and semi-aquatic species.

1.3 eDNA Metabarcoding

Environmental DNA (referred to as eDNA) is a relatively new concept that is defined as DNA shed from individuals (present in skin cells, urine, blood, faeces, saliva, etc…) that has been left behind in the environment, both modern and ancient (Pawlowski et al., 2020; Thomsen & Willerslev, 2015; Deiner

et al., 2017, Taberlet et al., 2012, Ficetola et al., 2008). eDNA is defined as DNA extracted from the environment, not from obvious biological material, such as faecal, blood and saliva samples, which are not defined as eDNA (Thomsen and Willerslev, 2015). eDNA studies have been able to detect both extant and extinct species which allows for both monitoring current biodiversity and gain information on past communities (Thomsen & Willerslev, 2015). Pawlowski et al. (2020) explain how eDNA should be defined with a 'two-level terminology' that will describe the methodology used and target taxa. The first level would provide information of how and where the DNA is extracted from: water eDNA, sediment eDNA, air eDNA and soil eDNA. The second level would provide information of the target taxonomic group that is to be amplified and sequenced: fish eDNA, invertebrate eDNA, bacteria eDNA, mammal eDNA, ect. For this study water eDNA was collected to identify mammalian eDNA. The focus for the application of eDNA has largely been on the detection of aquatic species, native and invasive, due to the direct shedding of DNA in the water environment making it simple to collect in theoretical comparison to terrestrial species (Rees et al., 2014; Takahara, Minamoto & Doi, 2013). These early studies were successful in detecting invasive aquatic species in fish, *Lepomis macrochirus,* (Takhara et al., 2013), reptiles, *Python bivittatus* (Piaggio et al., 2013) and amphibians, *Lithobates catesbeianus* (Dejean et al., 2012). The sensitivity of eDNA meant species of conservation interest were also being detected using eDNA when traditional methods were failing to do so which can allow for conservation decisions to be made quickly for invasive species or species of conservation concern (Thomsen et al., 2012). DNA has been shown to persist ~two weeks in laboratory settings and 48 hours to one month in aquatic and marine environments (Ficetola et al., 2008; Williams et al., 2018; Collins et al., 2018).

eDNA studies originated by focusing on detection of single species by using species-specific primers (Hunter et al., 2015; Dejean et al., 2015; Ficetola et al., 2008), however the use of universal primers, such as MiFish (Miya et al., 2015) and MiMammal (Ushio et al., 2017), allows for multi-species detection, expanding the value of eDNA (Seymour, 2019). eDNA metabarcoding is a molecular biodiversity monitoring technique that can successfully detect multiple species at once from DNA extracted from water, soil, air or from blood-sucking insects (the latter referred to as iDNA) using next-

generation sequencing (NGS) (Deiner et al., 2017; Pawlowski et al., 2020). NGS is a high throughput, low-cost, multi-sample sequencing technology which compared to real-time polymerase chain reaction (qPCR) is highly beneficial to community studies as NGS does not require specific primers for species detection (Liu et al., 2012).

For biological biodiversity monitoring the use of eDNA has been established as a cost effective and rapid methodology. With most early eDNA research focusing on aquatic species due to the evident DNA presence within the water systems (McDevitt et al., 2019), research into semi-aquatic and terrestrial mammals are yielding promising results on multiple taxa identification as well as detection for rare, elusive, and invasive species (Broadhurst et al., 2021; Sales et al., 2020a; Sales et al., 2020b; Leempoel et al., 2020; Harper et al; 2019; Lyet et al., 2021, Kim et al., 2020; Ushio et al., 2017). Due to the regularity of mammal species' contact with water, DNA will be captured in water samples. This has also been found to be true for animals which do not regularly contact water sites (Williams et al., 2018, Sales et al., 2020a, Broadhurst et al., 2021). Semi-aquatic animals will obviously spend longer amounts of time within the water and have a significantly higher chance of being detected (Harper et al., 2019). Despite successful semi-aquatic and terrestrial mammalian species detection using water eDNA there is a possibility of missing species with one time sampling therefore a 'multi sampling approach' has been recommended to accurately detect species with behaviours or life stages which might cause them to be missed by the first sampling session (Ficetola et al., 2008; Thomsen et al., 2012). A 'multi sampling approach' would see researchers sample temporally, either seasonally or yearly, to be able to follow population trends and record elusive species that may have been missed due to breeding, distance, or absence during a sampling time. This recommendation of temporal or 'multi sampling approach' has been similarly mirrored in mammalian eDNA studies, more recent studies have been providing reliable results from water samples to successfully detect semi-aquatic and terrestrial mammals but an emphasis on multi sampling is imperative due to species' behaviour and ecologies (Sales et al., 2020a; Sales et al., 2020b; Ushio et al., 2017; Harper et al., 2019; Leempoel et al., 2020; Lyet et al; 2021). Using eDNA to determine the abundance of a species has been used

(Thomsen et al., 2012), however both false positives (contamination) and false negatives (species masking and incomplete databases) may occur (Lodge et el., 2012; Roussel et al., 2015).

Animal behaviour, habitat preference and group size may also affect sequence/read count. Animals with large mass have been seen to have higher DNA read counts as it is hypothesised that large mass (as seen with Bears) will equal more DNA shed (Lyet et al., 2021). Group size can also affect DNA degradation rates so species can be detected for longer (Williams et al., 2018). Though, the consensus regarding DNA degradation rates is that positive detections of species indicate recent shedding from an animal, rather than DNA that has been in the environment for a while, thus implying that eDNA can detect invasive or rare species much earlier than some traditional methods (Williams et al., 2019; Thomsen et al., 2012). DNA degradation is a topic widely discussed by eDNA researchers based on laboratory experiments following degradation of cells in ~2 weeks (Williams et al., 2018; Thomsen et al., 2012) but fail to account for uncontrolled factors. Three broad categories of DNA degradation in environments have been described: DNA molecule characteristics, abiotic factors, and biotic factors (Barnes et al., 2014). The length of a DNA fragment can affect its binding ability to other particles which would extend its life (Romanowski et al., 1993). Abiotic factors such as UV (Sutter & Kinziger, 2019), temperature, pH (Seymour et al., 2018), oxygen levels and salinity can also affect DNA degradation rates. High temperatures can denature the molecule and UV light can also increase degradation rate (Barnes et al., 2014; Kessler et al., 2020).

1.4 eDNA and carnivores

Despite mammalian detections with eDNA being successful, studies often have low detections and/or sequence reads from carnivorous mammals (Broadhurst et al., 2021; Lyet et al., 2021; Sales et al., 2020; Harper et al., 2019). This could be due to inability of the primers to amplify certain carnivorous species, for example a bear specific primer (MiMammal-B) was used to ensure bear amplification due ineffective amplification using MiMammal seen in a preliminary study (Ushio et al., 2017). There is a

lack of research into carnivore specific eDNA studies, most studies using 'eDNA' are taking DNA directly from a known source (footprint and saliva) and not from the environment (Franklin et al., 2018; Wheat et al., 2018), or are species specific (Rodgers et al., 2015; Wilcox et al., 2021). Although successful, these studies are using single-species PCR to amplify the DNA, so we are unable to contribute towards understanding why metabarcoding studies are detecting low frequencies of carnivore sequences.

Otters are one such species that is frequently missed (cases of false negatives have been reported; Sales et al., 2020a; Harper et al., 2019; Thomsen et al., 2012). Lyet et al. (2021) also report difficulty in detecting carnivores, specifically felids. Carnivores with strict carnivorous diets (felids) had a lower detection rate than more omnivorous carnivores such as mustelids and canids, although it is theorised the results may vary in different seasons depending on what food is more available. Lyet et al. (2021) recorded higher eDNA detection rates for bears, which correlates with the omnivorous diet and body size. Both semi-aquatic and terrestrial carnivores are being detected meaning that DNA from carnivores who infrequently come in contact with the water is being identified with studies further demonstrating eDNA ability to reflect terrestrial communities (Broadhurst et al., 2021).

As there have been difficulties in detecting carnivores in some mammalian eDNA research, Lyet et al. (2021) suggest higher volume of water would be needed to successfully detect all species in the environment, however this would increase overall costs. Traditional methods frequently outperformed eDNA when focusing on carnivores: this could be due to most carnivores living solitary lives with large home ranges (Broadhurst et al., 2021). We would assume semi-aquatic carnivores to have large amount of sequence reads when using eDNA, but otter has frequently been recorded on camera traps with low or non-existent reads being detected from water samples (Sales et al., 2020a; Harper et al., 2019; Thomsen et al., 2012).

1.5 Occupancy modelling

As all monitoring methods are not able to accurately detect all species all the time, occupancy models are a useful approach to account for imperfect detections to provide species distribution probabilities (Mackenzie et al., 2002). Rare, elusive, and threatened species which reside in small populations may only be detected a small handful of times when using any monitoring method. Occupancy modelling has been successful in monitoring populations of the vulnerable sloth bear, *Melursus ursinus* in India (Das et al., 2014) and lemurs in Madagascan forests, traditionally difficult areas to monitor species, (Keane et al., 2012) and identify areas for conservation for the critically endangered Balkan lynx, *Lynx lynx balcanicus (*Melovski et al., 2020). Occupancy modelling can provide valuable information on population trends and areas in need of focus for endangered species. By accounting for imperfect detection this prevents underrepresentation of species presence that can bias the relationship between species occurrence and habitat variables which in turn can influence conservation managers decisions to help streamline legislation and funds, which are lacking within conservation (Lindermayer et al., 2012; MacKenzie et al., 2006).

Similarly, to other traditional monitoring methods, eDNA presence and absence data can be used to estimate detection probabilities of each species (Sales et al., 2020a). The high sensitivity of eDNA detections when compared to traditional methods can improve detection probabilities and site occupancy estimates, thus increasing focused conservation efficiency (Schmelzle et al., 2016; Schmidt et al., 2013).

1.6 Mammals in Spain

111 recorded species of mammals reside in Spain comprising 48% of all verified mammals within Europe (IUCN, 2013). The orders found in Spain are Rodentia, Lagomorpha, Erinaceomorpha, Soricomorpha, Chiroptera, Cetacea, Carnivora and Artiodactyl. 11% of these species are considered Near Threatened (NT), 9% are Vulnerable (V), 4% are Endangered (EN) and 3% are Critically

Endangered (CR) (IUCN, 2013). The biggest threat to native mammals in Spain is invasive species. 22 vertebrate species have been identified within the Iberian Peninsula as invasive, with mammal species making up 8.51% of them (Capdevila-Argüelles and Zilletti, 2001).

1.7 Carnivores in Spain

There are four threatened carnivorous species in Spain: the Iberian lynx, *Lynx pardinus* (EN) (Rodríguez and Calzada, 2015), Iberian grey wolf, *Canis lupus signatus* (V), Eurasian otter, *Lutra lutra* (NT) (Roos et al., 2015) and European mink, *Mustela lutreola* (CR) (Maran et al., 2016). There are 17 carnivorous species found in Spain (Table 1). It is difficult to have a true positive detection for wolves due to the similarity in the genetic sequence between wolves and domestic dogs, *Canis lupus familiaris.* The reported hybridisation between *C. lupus familiaris* and C*. lupus signatus* within the Iberian Peninsula adds another level of difficulty distinguishing the species due to the exchange in gene flow (Pires et al., 2017). Despite the initial challenges in detecting wolves there has been successful eDNA detection of other carnivorous mammalians: raccoons (*Procyon lotor*, Leempoel et al., 2019), mink (*Neovison vison* and *Mustela lutreola*), badgers (*Meles meles*), foxes (*Vulpes vulpes*, Broadhurst et al., 2021; Sales et al., 2020a) and felids (Lyet et al., 2021) which are all species that occur within Spain. The American mink (*Neovison vison*) is an invasive carnivore introduced to Europe in the 1920s during the fur industry boom. Feral populations were formed from escapees and intentional release (Podra and Gomez, 2017). Since then, *N. vison* has become a major competitor to the native mink, *M. lutreola. N. vison* is a generalist carnivore who predates on similar prey to *M. lutreola*, which has led to competitive exclusion (Santulli et al., 2014). Early detection is a main strategy for control of alien species, if eDNA is successful at detecting carnivorous species it could become a powerful tool for eradication programs (Bonesi and Palazon, 2017). As discussed already traditional methods have a high chance of misidentification so eradication programs not utilising DNA sequencing can be costly and unnecessary (Browett et al., 2020). A study into released *M. lutreola* found that predation accounted for 76% of mortalities which likely came from one lone male *N. vison.* This shows the disastrous consequences *N.*

vison can have on *M. lutreola* reintroduction programmes. Therefore, early detections using DNA would be important for recovery programmes and will allow for eradication efforts to start before a population has been established (Broadhurst et al., 2021; Põdra et al., 2013). *M. lutreola* is a specialised hunter and is already experiencing pressures from climate change, habitat destruction and declines in prey (e.g., crayfish (*Astacus astacus*); Maran and Henttonen, 1995; Maran et al., 2011).

Table 1. List of carnivores found in Spain with their IUCN status and EU conservation status of described species in Spain. Unknown; no assessment can be made, Bad; species is not viable long-term, Poor; not as critical as bad but still requires conservation, Good; species is viable and self-maintaining.

Species	**Common name**	**IUCN status**	**EU conservation status**	**IUCN Author**
Felis silvestris	European wildcat	EN	Unknown	Yamaguchi et al., 2015
Lynx pardinus	Iberian lynx	EN	Poor	Rodríguez & Calzada 2015.
Genetta genetta	Common genet	LC	Good	Herrero & Cavallini, 2007
Herpestes ichneumon	Egyptian mongoose	LC	Good	Do Linh San et al., 2016
Canis lupus signatus	Iberian wolf	LC	Poor	Boitani et al., 2018
Vulpes vulpes	Red fox	LC	-	Hoffmann & Sillero-Zubiri, 2021
Ursus arctos arctos	Cantabrian b. bear	LC	Poor	McLellan et al., 2018
Procyon lotor	Raccoon	LC	-	Timm et al., 2016
Lutra lutra	Eurasian otter	NT	Good	Roos et al., 2015
Martes foina	Beech marten	LC	-	Abramov et al., 2016
Martes martes	European pine marten	LC	Poor	Herrero et al., 2016
Meles meles	Eurasian badger	LC	-	Kranz et al., 2016
Mustela erminea	Stoat	LC	-	Reid, Helgen & Kranz, 2016
Mustela lutreola	European mink	CR	Bad	Maran et al., 2016
Mustela nivalis	Least weasel	LC	-	McDonald et al., 2019
Mustela putorius	European polecat	LC	Good	Skumatov et al., 2016
Neovison vison	American mink	LC	-	Reid, Schiaffini & Schipper, 2016

1.8 Sampling area, aims and collaboration

For this study, a collaboration was established between the McDevitt lab group from the University of Salford, the Vincent Wildlife Trust (VWT) and WildCRU (University of Oxford) to create a comparative study of monitoring methods (eDNA metabarcoding and camera traps) when monitoring native European mink (*M. lutreola*) and the invasive American mink (*N. vison*) in a north-eastern area of Spain. To test both the validity and accuracy of eDNA metabarcoding, a direct comparison was made

to camera traps that were set up at the same sampling locations. 10 days of camera trap data was compared to 10 eDNA samples from each site, which also contributed to evaluating the effort needed to accurately represent mammalian communities when using eDNA within a temporal study. The eDNA samples were taken over two sampling periods, 10 days apart, which was supposed to give more validity to our occupancy modelling and account for animals that may be missed in one sampling session but is present in the area following a small-scale version of the multi sampling approach (Ficetola et al., 2008). Despite VWT specifically looking for both mink species, the samples taken allowed for a broader comparative study in detecting and comparing the wider mammalian community using eDNA and camera traps. This study focused on mammalian carnivores (due to the difficulty in detecting them using eDNA as outlined above) based on the total 17 carnivores found in Spain (Table 1).

2. Materials and methods

2.1 Study area

This study was run in conjunction with the VWT and WildCRU in the north-eastern area of Spain. The sampling area was split in to five 10 x 10 km squares, based on the Universal Transverse Mercator grid reference system, hereby known as UTM squares, with 5 points being sampled within each UTM square, 25 sites in total (Figure 1.). Sampling was conducted with 10 different rivers within the UTM sites. UTM1 consisted of rivers Tiron, Ea-Tiron and Oja, UTM2 consisted of Ebro and Zadorra, UTM3 rivers were Ebro and Najerilla, UTM4 consisted of rivers Balsa Channel, Zadorra Lbaia and Algeria with UTM5 only consisting of river Leza. Five samples will also be taken from an undisclosed mink enclosure to be used as positive controls.

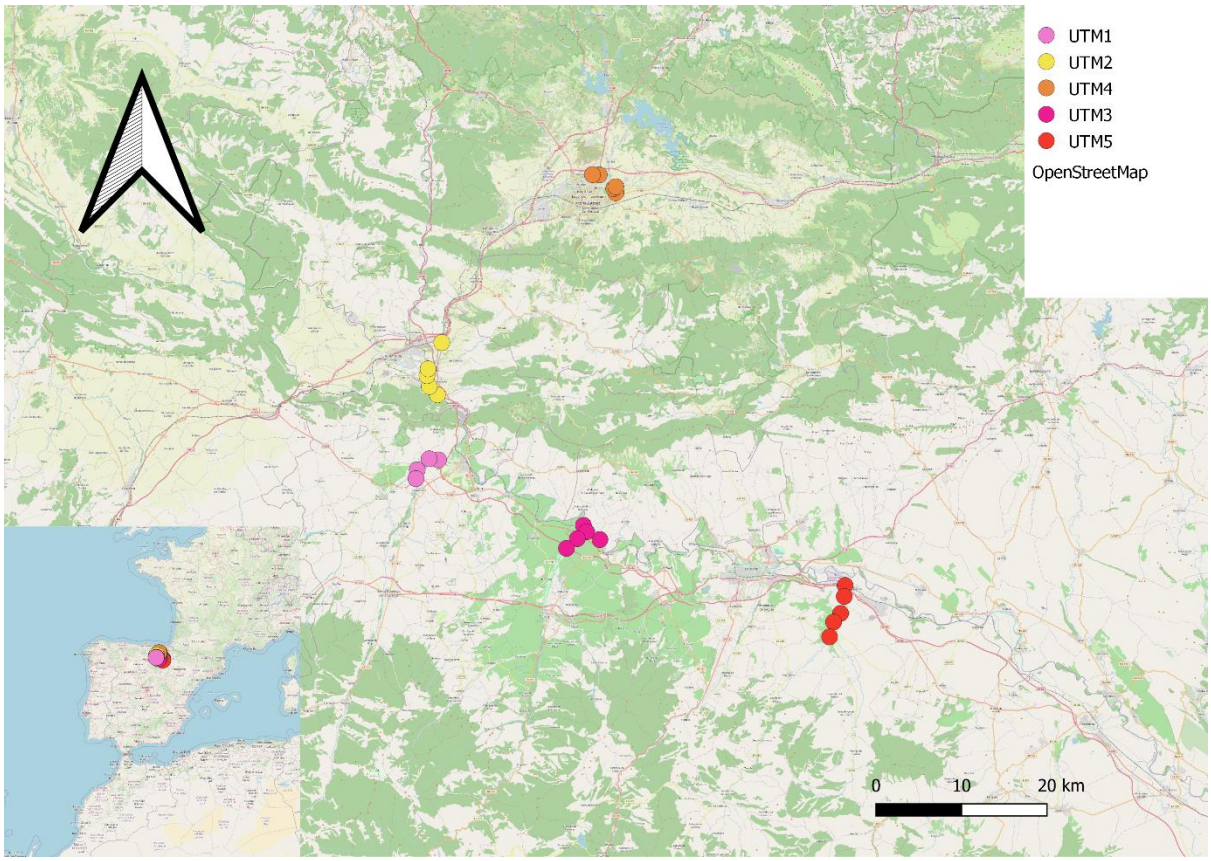


Figure 1. Arial map of the study site in north-eastern Spain with the five UTM sample sites. Bottom left map shows the area of Spain where the study was conducted. Maps created with QGIS 2020.

2.2 eDNA field sampling

Fieldwork was conducted by the VWT from 14th of October to the 28th of October 2019 for a study of *M. lutreola* detection. Each UTM square was sampled twice, starting on the 14th of October 2019 and the repeated 10 days later. Water samples were collected using 500ml sterile bottles, with 5 replicates taken at each site 5-10 meters from each other, following the protocol defined in Broadhurst et al. (2021) (Figure 2). Samples were collected at the water's edge, avoiding touching water apart from a sterile, gloved hand of the researcher. No abiotic factors were recorded for this study; however, it was noted that there was heavy rainfall between the two sampling sessions. At the beginning and end of each day a 500ml bottle of distilled water was opened for 30 seconds and then filtered, to be used as a field blank which can account for any contamination. The samples were collected and filtered in the field, using 0.45µm Sterivex filters, then refrigerated for 2 days before freezing in Spain. Frozen samples were sent to the University of Salford in cool boxes packed with ice to be extracted in a specialist eDNA laboratory. A total of 25 locations where sampled (Figure 3).

Figure 2. Collaborator Andrew Harrington collecting a water sample with a 500ml sterile bottle at one of the sampling sites in Spain.

Five samples from an undisclosed mink enclosure where also taken to be used as positive controls in the laboratory step. These samples were collected following the same protocol with a negative control

taken at the same time. A total of 264 samples were collected which included 240 field samples, 5 samples from the mink enclosure and 19 field blanks. Two field blanks were missed on the 17th of October and 10 replicates were missed from the 17th of October at Rivers Balsa Channel and Zadorra Lbaia within UTM3.

2.3 Camera Traps

50 camera traps were deployed in total along the 25 sampling points, two cameras at each point pointing towards the water. Two different models were used, Bushnell Trophy Cam HD and Browning Strike Force HD Pro X. Each camera was set to record still images over 10 days, which would coincide with the eDNA sampling. The cameras were fastened to stakes or trees and baited with sardines which was replenished five days later. The positioning and use of bait was to try and accurately record images of both *M. lutreola* and *N. vison*. Teams from the VWT went through the camera trap image data to identify any species captured. Excel spreadsheets were created of the findings to be later analysed.

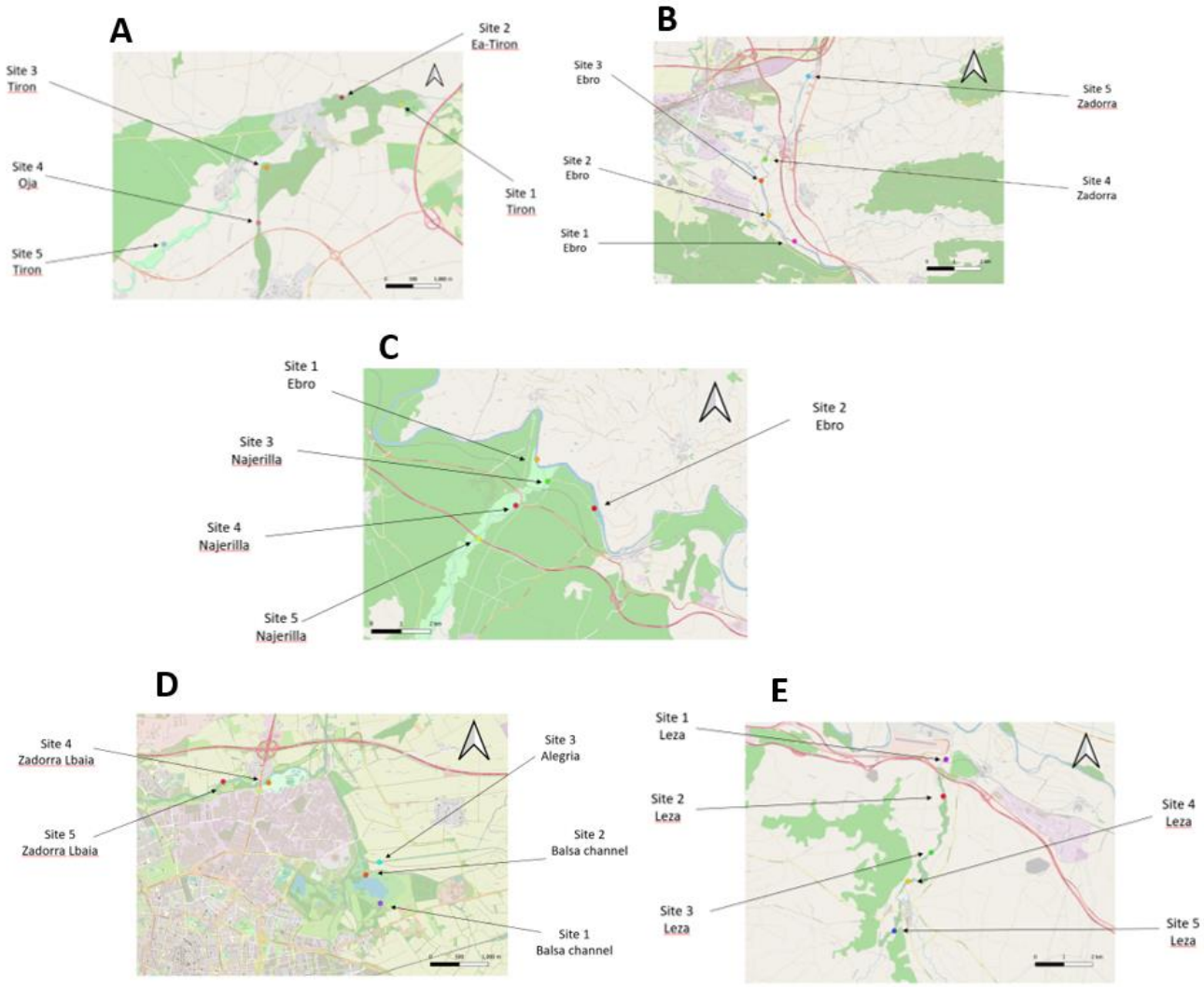


Figure 3. Maps of the 5 UTM squares with the 5 rivers sampled. **A)** UTM square 1, rivers Tiron, Ea-Tiron and Oja. **B)** UTM square 2, rivers Ebro and Zadorra. **C)** UTM square 3, rivers Ebro and Najerilla **D)** UTM square 4, rivers Balsa channel, Alegria and Zadorra Lbaia. **E)** UTM square 5, river Leza

2.4 eDNA extraction

DNA extractions were carried out over the months of July and August of 2020. The University of Salford houses a specialised eDNA extraction room which is sterilised daily with UV lights for 2 – 6 hours. Full body protection is worn to prevent potential contamination from the extractors. DNA was extracted from the filters using the mu-DNA water Protocol v. 0219 (Sellers et al., 2018). At the 'Wash' stage 600µl of solution was added to the spin column, rather than the proposed 500µl due to previous University of Salford researchers' recommendations. The Lysis solution, Flocculant solution, Binding

solution, Wash solution and Elution buffer were made by myself and other colleges in the eDNA laboratory. To account for in laboratory contamination one laboratory blank, containing only buffer, was incorporated into the extraction process each day, for a total of 12 extraction blanks, meaning 252 samples were extracted in total. Field blanks were extracted first, followed by the field samples and finally the captive mink samples to limit possible DNA contamination. As contamination risk is high extensive cleaning was taken at each extraction step as prevention (work stations were cleaned with 10% bleach and 70 % ethanol and equipment was placed in a UV Stratalinker® before and after use).

Eluted DNA was frozen at -20 °C before being thawed for the amplification stage. The extracted eDNA was amplified using the MiMammal 12S primer set (MiMammal-U-F, 5'-GGGTTGGTAAATTTCGTGCCAGC-3'; MiMammal-U-R, 5'-CATAGTGGGGTATCTAATCCCAGTTTG-3'; Ushio et al., 2017) targeting a ~170 base pair (bp) amplicon from a variable region of the 12 rRNA mitochondrial gene with six base sample-specific multiplex identifier (MIDs) tags. The MID tags contained wobble / degenerate bases to increase variation; these bases would be discarded in the bioinformatic stage. The total samples (including blanks) where split into four libraries for the PCR stage. Each PCR reaction was replicated 3 times per sample. Five PCR positive controls were incorporated to account for tag switching due to the non-target species not occurring in the north-eastern area of Spain (the control contained 0.05ng/µL faecal sample from Northern muriqui, *Brachyteles hypoxanthus*) and four negative controls (ddH_20) per PCR plate (Broadhurst et al., 2021). 20µL of product, containing 2 µL of extracted DNA, 2µL of forward and reverse MiMammal primers (1µL of forward, 1µL of reverse), 16 µL of premixed PCR master mix containing 5.84µL of H_20, 0.16µL of Bovine serum albumin (BSA) and 10µL of Amplitaq, was loaded into the PCR machine. After the 10 minutes of initialisation at 95°C the thermal cycle profile ran 40 times denaturing at 95°C for 30 seconds, annealing at 60°C for 45 seconds, elongating at 72°C for 30 seconds. The final elongation ran at 72°C for 5 minutes to ensure and single stranded DNA was fully extended before cooling at 10°C. 1.5% agarose gels were prepared to confirm successful amplification: 2 µL of DNA was stained with 1

µL loading buffer for visualisation and run alongside 3µL of HyperLadder 1kb which was electrophoresed for one hour at 70V. PCR triplicates are then pooled together into four libraries. A left-sided size selection targeting product under 245~ base pairs was performed using 1.1x Agencourt AMPure XP (Beckman Coulter) and Dual-Index adapters (Illumina) were added to each library using KAPA HyperPrep kit (Roche). Libraries where then quantified using NEBNext qPCR quantification kits to ensure DNA concentration was between 8 – 12nM. The four libraries were run on over two sequencing runs: library 1 and 2 in the first run and library 3 and 4 in the second run. 120 samples, 10 field blanks (nine in library 2), eight extractions blanks, eight negative controls (nine in library 2), 10 positive controls, and four replicates of captive samples, were run equalling a total of 380 total samples. Each library was run at a final molarity of 9 pM on a multiplex Illumina Miseq v2 Reagent V2 Kit paired-end reads (as detailed in Sales 2020a and Broadhust et al., 2021).

2.5 Bioinformatics analysis

The final libraries read data quality was first tested using FastQC (Andrews 2010). The libraries were individually run through a bioinformatics pipeline using OBITools metabarcoding package (Boyer et al., 2016). Forward and reverse pair-end sequences were then aligned using illumine pair-end, and dataset was demultiplexed to sort sequences by sample location using ngsfilter. Sequence length was filtered to consist of reads between 160 – 190 base pairs due to the target length being ~170bp; obigrep was used to remove reads with ambiguous bases. Obiunique clustered identical sequences and chimera removal was done using vsearch (Rognes et al., 2016) using uchinme-denovo (Edgar et al., 2011). Reads were then clustered with SWARM (Mahé et al. 2015) and a taxonomic assignment conducted using ecotag (Sales et al., 2020; Broadhurst et al., 2021). An output of CSV files contained the molecular operational taxonomic units (MOTUs) with a taxonomic assignment. Additional filtering steps were added to remove sequencing errors to avoid false positives (Broadhurst et al., 2021). The rate of tag-jumping between amplicons (Schnell et al., 2015) was calculated by dividing the total number of positive control (PC) reads (*B. hypoxanthus*) found in 'real' eDNA samples by the total

number of PC reads in the PC samples. The percentage frequency was then deducted from every sample and the PC reads where removed using R-Studio v4.0.0 (R Core Team, 2020). In total 0.04%, 0.0001%, 0.03% and 0.008% of reads were deducted from each library respectively (Table. 2). To account for potential contamination, the maximum number of reads recorded in the negative controls (including field blanks, extraction blank or distilled water used in the PCR reaction) were removed from every sample. Lastly, non-target MOTUs were removed from the data set. This includes non-mammal species, human and domestic species. This ultimately led the decision to remove potential 'Boar' and 'Wolf' reads from the data set, although these species are both native in north-eastern Spain, the sequences are indistinguishable from that of domestic pig and domestic dogs at this short 12S fragment. Samples with <5 reads overall were also excluded from the final data set. MOTUs that were identified to a >0.97 match to a species in the custom UK database were included (Broadhurst et al., 2021 & Sales et al., 2020a). A decision was made to include the Southern water vole (*Arvicola sapidus*), genet (*Genetta servalina*), Granada hare (*Lepus granatensis*), European harvest mouse (*Micromys minutus*) and the aquatic mole (*Talpa Aquitania*) despite the match being below the defined threshold above (0.93, 0.95, 0.95, 0.94 and 0.94 respectively). The inclusion of these species was due to the high number of reads in the samples, similarly, seen in Lyet et al. (2021). These species had a low threshold due to incomplete databases which subsequently leads to species being missed as they are filtered out so were included in this study for community accuracies (Leempoel et al., 2020). Each species was checked with Genbank to ensure correct species identification.

2.6 Statistical analysis

R-Studio v4.0.0 (R Core Team, 2020) was the main analytical tool used to analyse the data. QGIS 3.13.3 (QGIS Development Team, 2021) was used to create the maps of the sampling locations. Coordinates collected by the VWT team at each sampling location in the field were imported on to a map within QGIS. An overall map was created of the capturing the whole sampling location (Figure 1) as well as individual maps of each UTM square (Figure 3).

iNEXT (Chao et al., 2014) was used to create species accumulation per site (Figure 5) for both sampling occasions and the combined sampling effort. A species accumulation was also created for carnivore detections by combining eDNA and camera trap data (figure 10A & B). rGGplot2 (Wickham, 2016) was used to create bubble charts which showed the number of reads recorded at each sampling location for the carnivore species.

The unmarked package (Fiske & Chandler, 2011) created occupancy and detection probabilities for carnivore detection using both eDNA and camera traps as a comparison method (Table 4). The decision was made to combine the two eDNA sampling session results together to get a more accurate overall picture of the mammalian biodiversity in the area. As the camera traps were deployed for a minimum of 10 days at each site it was decided to treat one day as one detection event compared to one eDNA replicate for a direct comparison, i.e. a unit of surveying efficiency is represented by one eDNA replicate and one day of camera trap surveying (Rich et al., 2016). The rationale behind this decision is that 10 days of camera traps and 10 replicates were recorded at each location so direct comparison would not be appropriate given the different sampling effort. MacKenzie et al. (2002) single species occupancy model was applied to the data where only presence and absence data was gathered from the eDNA replicates and camera trap dates (MacKenzie et al., 2017). Following Sales et al. (2020a) analysis with the assumptions of the carnivore species presence or absence being a constant within the sampling period then it represents a 'true' occupancy status at the time of sampling. The unmarked package was used to create occupancy data for the multi-site occupancy models for each carnivore species using both eDNA data, camera trap data and the data combined.

3. Results

3.1 eDNA species detection and filtered reads

The MiSeq sequencing run produced 4762101, 5253351, 5293162 and 3823069 reads per library respectfully, totalling 19131683 reads overall. After filtering the final read count reduced to 5032263 (Table 2). A total of 24 species were identified from the overall sampling efforts. 18 species were detected in the first sampling session and 22 identified in the second sampling session, only *Capreolus capreolus* and *Lepus granatensis* were not detected in the second sampling session (Table 2 and Figure 4).

Table 2. Number of reads after each filtering step to remove percentage of potential tag-switching, non-target species and potential contaminations for each library.

	Library 1	**Library 2**	**Library 3**	**Library 4**
Tag switching percentage	0.04632	0.00011	0.03125	0.00763
Reads before filtering	4762101	5253351	5293162	3823069
Reads after tag switching % removal	4495235	5030138	4990828	3637988
Reads after positive control removal	4493980	5030112	4990823	3637986
Reads after negative control removal	4249829	4781590	4602351	3317367
Reads after selecting mammals only	3494214	4213161	4205877	2580022
Reads after human reads removal	1967269	1947145	2899004	2155556
Reads after removing domestic animals (*Sus, Bos, Felis, Ovis, Canis* and *Capra*)	586877	624674	2098206	1821916
Reads with >0.98 identification and less than 5 reads removal	277094*	426140	592177	914981
Reads with identified species under 0.98 identification**	564460	604291	2058926	1804586

*Manually removed 18 reads from Library 1 due to potential contamination of European water vole, *Arvicola amphibius. Original final reads 277112.*
***Library 1 and 2 species with 0.97 identification, Library 3 species with 0.95, 0.94 and 0.93 and Library 4 species with 0.94 and 0.93 identification.*

Overall eDNA was able to detect species from most conservation status (according to the IUCN global classification). 19 least concern species (*Vulpes vulpes, Meles meles, Mustela nivalis, Genetta servalina, Castor fiber, Apodemus flavicollis, Rattus norvegicus, Mus musculus, Glis glis, Myodes glareolus, Mus spretus, Apodemus sylvaticus, Micromys minutus, Crocidura russula, Sorex araneus,*

Lepus granatensis, Cervus elaphus, Capreolus capreolus and *Dama dama*), one vulnerable species (*Arvicola sapidue*), two near threatened species (*Lutra lutra* and *Oryctolagus cuniculus*) and one critically endangered species (*Mustela lutreola*) (Table 3). One species (*Talpa Aquitania*) does not have a status as it was only recently described (Nicolas et al., 2016).

Five different orders were identified. Rodentia was the order with the greatest number of species detected (10). The majority of the Rodentia are categorised as least concern with one species classified as vulnerable, *Arvicola sapidue*. The next largest order detected was Carnivora with six identified species. Artiodactyl and Eulipotyphla orders both had three species, and two species were identified in the Lagomorpha order (Table 3).

Table 3. Species detected using eDNA over two sampling periods. 1 is given if the species is detected in at least one out of the replicates taken, 0 is given if the species is undetected. IUCN status (LC = Least concern, NT = near threatened, VU = vulnerable, CR = critically endangered).

Order	Scientific name	Common name	IUCN status	Sampling session 1	Sampling session 2
Carnivora	*Vulpes vulpes*	Red fox	LC	1	1
	Mustela lutreola	European mink	CR	1	1
	Meles meles	European badger	LC	1	1
	Lutra lutra	Eurasian otter	NT	1	1
	Mustela nivalis	Least weasel	LC	0	1
	Genetta servalina	Genet	LC	0	1
Rodentia	*Castor fiber*	Eurasian beaver	LC	1	1
	Apodemus flavicollis	Yellow-necked mouse	LC	1	1
	Rattus norvegicus	Brown rat	LC	1	1
	Mus musculus	House mouse	LC	1	1
	Glis glis	Edible dormouse	LC	0	1
	Arvicola sapidue	Southern water vole	VU	1	1
	Myodes glareolus	Bank vole	LC	1	1
	Mus spretus	Algerian mouse	LC	1	1
	Apodemus sylvaticus	Long-tailed wood mouse	LC	1	1
	Micromys minutus	Eurasian harvest mouse	LC	1	1
Eulipotyphla	*Crocidura russula*	Greater white-toothed shrew	LC	1	1
	Sorex araneus	Common shrew	LC	0	1
	Talpa aquitania	Aquatic mole	NA	0	1
Lagomorpha	*Oryctolagus cuniculus*	European rabbit	NT	1	1
	Lepus granatensis	Granada hare	LC	1	0
Artiodactyl	*Cervus elaphus*	Red deer	LC	1	1
	Capreolus capreolus	Roe deer	LC	1	0
	Dama dama	Fallow deer	LC	0	1

Figure 4. Species detection per sampling site using eDNA metabarcoding from sampling session one (pink) and sampling session two (blue).

Viewing the species accumulation curves for the two sampling sessions for all species, sampling session two was more successful in recording more species, as it captured 22 out of the 24 overall recorded species (Figure 5, Table 3). Visually assessing the accumulation graph for all species, the first sampling session would need an increased sampling effort to record all species. As a combined sampling curve was created to look at species recorded when combining both sampling session it has been decided to use both sampling sessions as a whole data set as an appropriate amount of sampling and species detected.

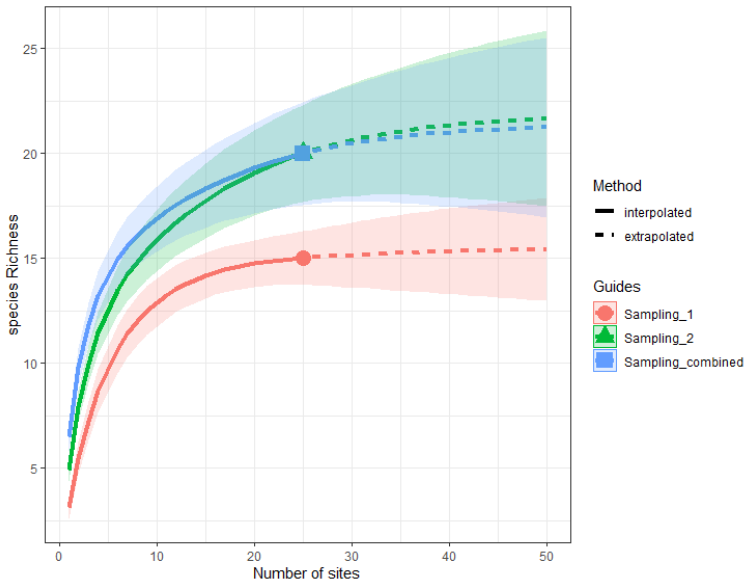


Figure 5. Species accumulation curve for all mammalian species identified using eDNA at each site.

Bubble charts for carnivore species detection over the two sampling sessions show differences in both species detected and amount of reads from each species (Figure 8 and 9). The initial observation is the number of species detected per session, with only four carnivores being detected in the first sampling session: red fox (*V. vulpes*), European mink (*M. lutreola*), badger (*M. meles*), and Eurasian otter (*L. lutra*), over 11 of the 25 sites (Figure 4 and Figure 8). Six species were identified in the second sampling session (Figure 4 and Figure 8), these include the four carnivores detected in sampling

session one plus the least weasel (*Mustela nivallis*) and genet (*Genetta servalina*). These six carnivores were detected in 13 of the 25 sites.

Multiple carnivore species were recorded at rivers Tiron 1 (two species), Ea-Tiron 2 (four species), Tiron 3 (four species), Ebro 1 (two species), Najerilla 5 (three species), Algeria 3 (four species), Zadorria Lbaia 4 (two species), Leza 2 (four species), Leza 3 (two species) and Leza 4 (three species) across both sampling sessions. The only carnivore species that were recorded in the same location on both sampling sessions were *V. vulpes* at site Tiron 3, *M. lutreola* and *M. meles* at site Algeria 3, all other readings were only gathered from one of the sampling sessions. (Figure 6).

The bubble charts also visually demonstrate the amount of sequence reads recorded in each sampling site, overall, more reads were gathered from sampling session two (Figure 6).

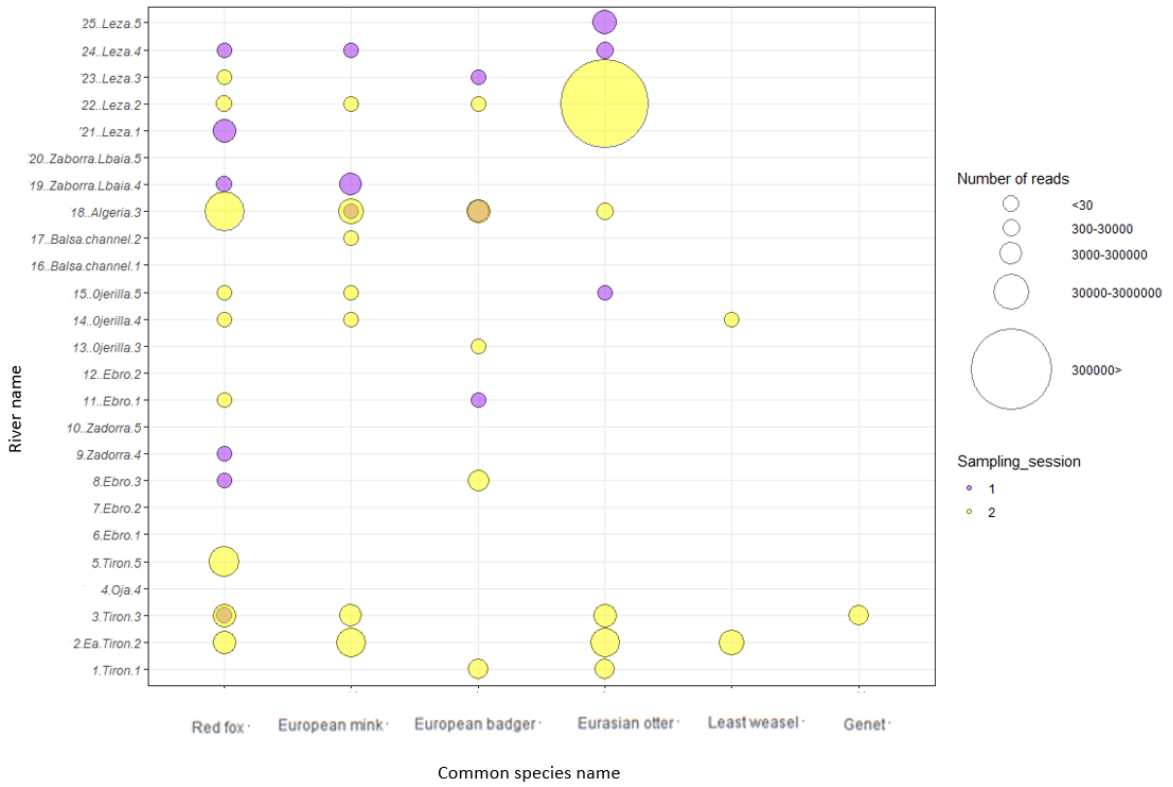


Figure 6. Bubble chart of eDNA sequence reads of identified carnivore species from both sampling sessions. Sampling sites 1-5 are withing UTM1, site 6 – 10 are withing UTM2, sites 11 – 15 are withing UTM3, sites 16 – 20 are within UTM 4 and sites 21 – 25 are within UTM5.

3.2 Camera trap species detection

10 days of camera trap images where analysed and the species identified by the VWT. Most of the animals identified could not be identified down to specific species level so were described as 'groups'. Three orders were identified from the animals named; Carnivora (eight groups), Rodentia (three groups), Artiodactyl (two groups) and an 'unsure' species group (for animals where the image was not clear enough to identify). Within the Carnivora order the groups are not as defined, for example there is three mink categories; American mink, European mink, and 'unsure' mink where species level was unable to be identified (Table 3).

Table 4. Species detected from 10 days of camera trap recording within the 5 UTM squares. 1 is given if the species is detected in at least one of the 10 days in each UTM square, 0 is given if the species is undetected. Unsure mink is a category created when the mink species could not be determined.

Order	Species detected	UTM1	UTM2	UTM3	UTM4	UTM5
Carnivora	Genet	1	1	1	1	1
	Marten	1	1	1	0	1
	American mink	0	0	0	1	0
	European mink	1	0	0	1	1
	Unsure mink	1	0	1	1	1
	Otter	1	1	1	0	1
	Fox	1	0	0	1	1
	Weasel	0	1	0	0	0
Rodentia	Small rodent (Rat/mouse/vole/shrew)	1	1	1	1	1
	Beaver	1	0	1	0	1
	Squirrel	0	0	1	0	1
Artiodactyl	Boar	0	0	1	1	0
	Deer	1	0	0	1	1
	Unsure	1	1	1	1	1

Within the Carnivora order seven species/groups where identified: genet, marten, American mink, European mink, otter, fox, and weasel. ('unsure mink' is not classified as its own species as it would either by *M. lutreola* or *N. vison.*) Within the Rodentia order three groups of species are identified: small rodent, beaver, and squirrel. 'Small rodent' groups rats, mice, voles, shrews, and similar animals together. This is due to the difficulty in identifying small animals on the camera correctly. Two groups are identified in artiodactyl, deer, and boar. Deer and marten were not defined to species level.

3.3 Carnivore species detection

Overall, eight carnivore species were detected using both detection methods: European badger (*M. meles*), Eurasian otter (*L. lutra*), least weasel (*M. nivalis*), red fox (*V. vulpes*), genet (*G. servilas*), marten (*Martes*, only defined to genus level), European mink (*M. lutreola*) and American mink (*N. vision*). *M. meles* was only detected with eDNA and *N. vison* and *martes* were only detected with camera traps (Figure 7).

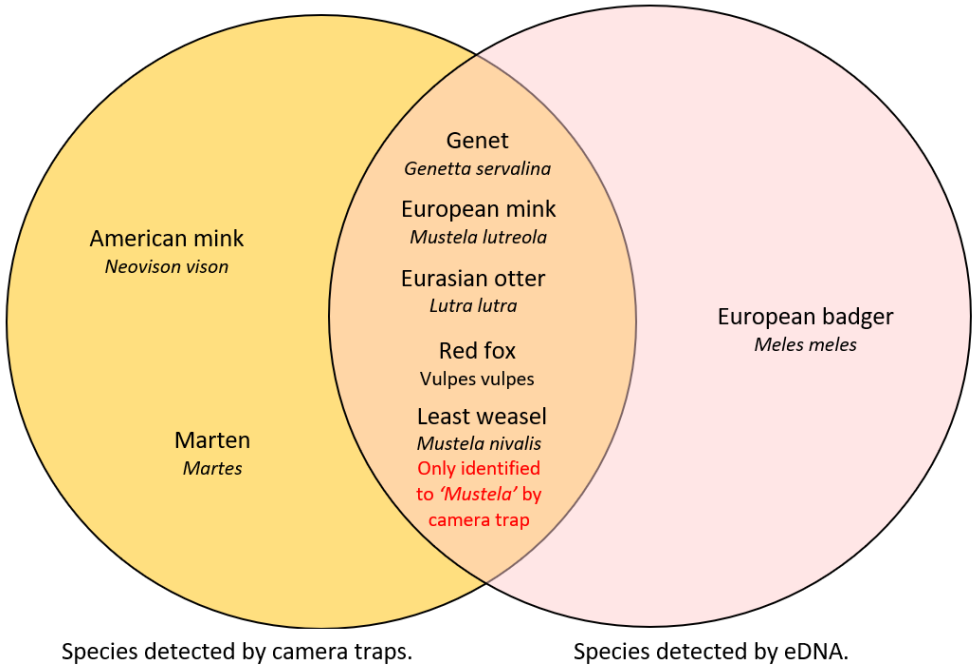


Figure 7. Venn diagram showing carnivore species detected using the different detection methods, eDNA and Camera trap.

On initial visual representation of the two methods (eDNA and camera traps) recording carnivore species, there is more data for the camera traps (Figure 9). *V. vulpes* was recorded at 14 sampling points (out of the 25) using eDNA and five sampling points using camera traps, with three of the locations detecting *V. vulpes* with both methods: 2. Ea-Tiron, 3. Tiron 2 (UTM1) and 23. Leza 3 (UTM5). *M. nivalis* was detected at two locations using eDNA and one location by camera traps, all three recordings were in different UTM squares. *L. lutra* was recorded at eight locations using eDNA and 11 locations by camera traps. *L. lutra* was recorded with both methods at five locations; 1. Tiron 1 (UTM1), 15. Najerilla 3 (UTM3), 22. Leza 2, 24. Leza 4 and 25. Leza 5 (UTM 5). *L. lutra* was recorded by at least one of our sampling methods in each UTM square.

M. lutreola was identified at nine locations via eDNA and 12 locations with camera traps. Both methods detected *M. lutreola* at five locations; 2. Ea-Tiron (UTM 1), 18. Algeria and 19. Zadorra Lbaia

1 (UTM4) and 22. Leza 2 and 24. Leza 4 (UTM 5). *M. lutreola* was detected in four out of the five UTM squares. *G. servalina* was only detected once with eDNA but was captured multiple times with the camera traps and recorded at 19 locations. *G. servalina* was identified via both methods at site 3. Tiron 2 (UTM 1). *M. meles* was only recorded by eDNA, at seven sites throughout all the UTM squares. *N. vison* and *Martes* was only recorded by the camera traps. *N. vison* was only recorded once, at site 19. Zadorra Lbaia 1 (UTM 4). *Martes* was caught via camera at 12 locations (Figure 9).

A)

E)

C)

D)

Figure 8. Group of images of carnivores identified in the sampling area in Spain. A) European mink, *Mustela lutreola* E) Genet, *Genetta servaline* C) European mink, *Mustela lutreola* D) Eurasian otter, *Lutra lutra*.

Figure 9. Species detection per sampling site using eDNA metabarcoding results from the combined sampling sessions equalling 10 replicates (green) and 10 days of camera traps (purple).

Carnivore species richness was compared using the combined sampling eDNA data and the camera trap data, 10 days of imaging compared to 10 samples taken. When samples are combined more species are detected and as the curve has not plateaued more sampling would be needed to record all carnivores (10B).

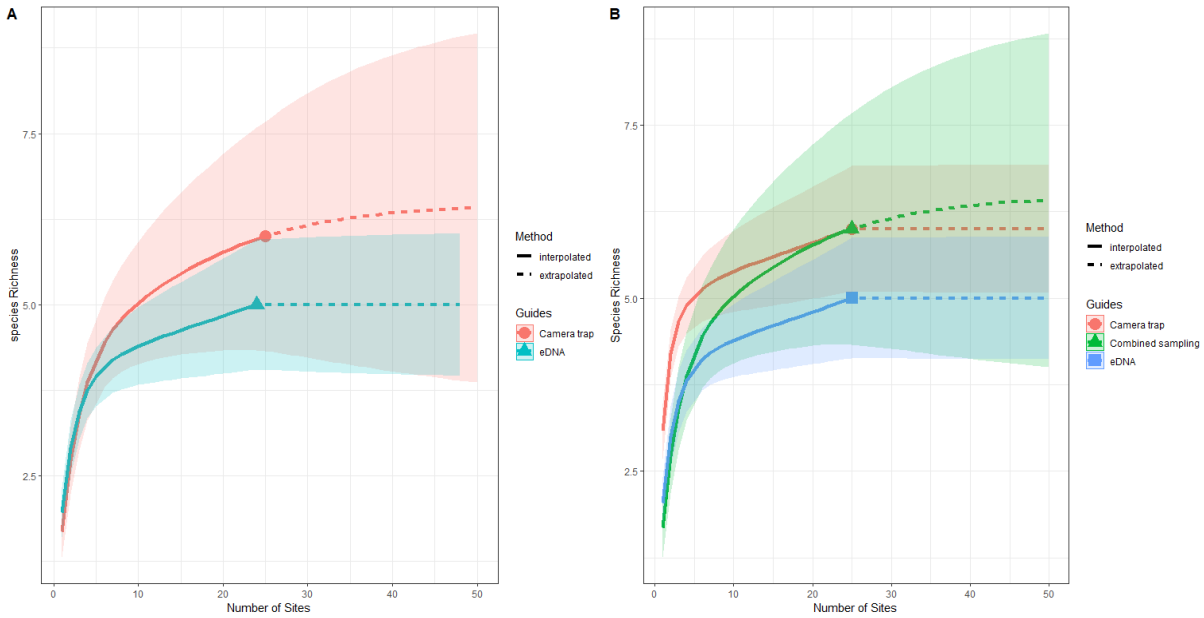


Figure 10. A) eDNA and camera trap species accumulation curves for Carnivore species **B)** eDNA, CT and combined sampling for carnivore species.

3.4 Carnivore occupancy and detection probability

Site occupancy and detection probability for each carnivore species was calculated using the total 10 eDNA replicates taken from each site compared to the 10 days of camera trap images per site using the unmarked packaged (Fiske & Chandler, 2011). Detection probabilities for carnivores were low continuously, all under 0.30 (Table. 5). *G. servalina* had an eDNA detection probability of 0 as it was only detected in one replicate out of the 250 taken. In comparison, the camera traps detection probability was 0.26 as it was detected in 48 out of the 250 days sampled. *V. vulpes* and *M. meles* also had low eDNA detection probabilities, 0.01 and 0.03 due to only being detected in 30 and 8 replicates, respectively. Although *V. vulpes* had 30 detections there was typically only one detection per site. *M. lutreola* and *M. nivallis* had similar detection probabilities of 0.12 and 0.10, respectively. However, *M.*

lutreola had a higher rate of detections (17 out of 250) compared to *M. nivallis* (3 put of 250). This again is due to where the detections were, *M. nivallis* three detections were from two different sites whereas *M. lutreola* detections were from nine different sites. *L. lutra* had the highest eDNA detection probability of 0.23, although only 17 detections were recorded most of them where from the same sites.

In comparison camera trap detection probabilities were either equal or slightly higher than eDNA overall. The largest difference is with *G. servalina*. Detection probability for camera traps ranged from 0 to 0.33 in comparisons to eDNAs detection probabilities ranging from 0 – 0.23. *V. vulpes* had the same detection probability for both sampling techniques (0.01), were as detection probabilities for *M. lutreola, L. lutra* and *M. nivallis* were all higher when using camera trap data (0.20, 0.27 and 0.11 respectively). However, the confidence intervals overlap considerably with all species with camera traps and eDNA detections. *M. nivallis* and *L. lutra* confidence intervals only had a 0.01 difference between the two methods and *M. lutreola* had a difference of 0.06. The largest difference was for *G. servalina* with a difference of 0.29. *M. meles* was not detected using camera traps and there is also no direct comparison for *Martes* and *N. vison* as they were not detected via eDNA. Camera trap detection probabilities for these species were 0.33 and 0, respectively. *Martes* was detected in 39 out of 250 days, within 12 of the sites. *N. vision* was only detected once on the camera traps (Table 5). As direct comparisons could not be made *M. meles*, *N. vison* and *Martes* were removed from Table 5.

Table 5. Estimated site occupancy and detection probabilities for each carnivore species, with 95% confidence intervals, from eDNA metabarcoding data from the total 10 replicates from each of the 25 sites and 10 days of camera trap data, individual and combined, created using unmarked (Fiske & Chandler, 2011).

		Occupancy			Detection probability		
Species	**Common name**	**eDNA**	**Camera Trap**	**Combined**	**eDNA**	**Camera Trap**	**Combined**
Vulpes vulpes	Red fox	1 (0 – 1)	1 (0-1)	1 (0-1)	0.01 (0.00 – 0.04)	0.01 (0.00 – 0.04)	0.02 (0.01 – 0.03)
Mustela lutreola	European mink	0.58 (0.19 – 0.89)	0.48 (0.25 – 0.72)	0.55 (0.33 – 0.79)	0.12 (0.05 – 0.26)	0.20 (0.11 – 0.32)	0.14 (0.08 – 0.21)
Lutra lutra	Eurasian otter	0.27 (0.12 – 0.49)	0.47 (0.28 – 0.67)	0.42 (0.28 – 0.58)	0.23 (0.13 – 0.38)	0.27 (0.18 – 0.37)	0.21 (0.16 – 0.29)
Mustela nivallis	Least weasel	0.13 (0.02 – 0.52)	0.1 (0.02 – 0.50)	0.11 (0.04 – 0.28)	0.10 (0.02 – 0.44)	0.11 (0.02 – 0.45)	0.13 (0.05 – 0.32)
Genetta servalina	Genet	0.99 (0 - 1)	0.77 (0.53 – 0.91)	0.41 (0.27 – 0.56)	0 (0.00 – 0.05)	0.26 (0.19 – 0.34)	0.24 (0.18 – 0.31)

4. Discussion

Although recent eDNA studies are showing success in detecting mammalian communities overall, the detection of carnivores using eDNA metabarcoding is a notable exception (Thomsen et al., 2012; Harper et al., 2019; Sales et al., 2020a; Leempoel et al., 2020; Broadhurst et al., 2021). This small-scale study has been successful at detecting 24 mammals, six belonging to the order carnivora. We show how temporal sampling increases the detection probabilities of this difficult-to-detect group with eDNA (Figure 3). eDNA metabarcoding can create a larger snapshot of the mammalian community present when compared to camera trapping alone, particularly when camera trapping is only focusing on specific species. eDNA metabarcoding provided comparable detections for most carnivore species and the adoption of both methods leads to an overall increase in detections at the landscape scale.

4.1 Mammalian community presence and absence

Here we see that the use of traditional methods detects a fraction of the mammals when compared to eDNA metabarcoding. eDNA metabarcoding identified 24 species from five different orders, while camera traps only identified three orders with 12 'categories' of animals detected. *N. vison* and *M. lutreola* were identified from the camera trap images by specialists at the VWT. A small handful of the categorised animals were defined to species level due to known historical data of species in the area, for example 'otter' can be defined to *L. lutra* due to only one species occurring in Spain but 'marten' cannot be identified to a specific species due to there being no distinguishing features being noted on the camera trap data and there being at least two species of marten in Spain (*M. martes* and *M. foina*). The difficulty in 'traditional' species detection was highlighted in the 'small rodent' category as this encompassed 'rat, mouse, voles and shrew'. The VWT researchers' decision to group small rodents together was due to the difficulty in identifying similar looking species which leads to a high level of misidentification, or, as the original study was focusing on *N. vison* and *M. lutreola*, there was not a necessity to accurately identify small rodents (Meek et al., 2017). To successfully detect and identify

small rodents, the addition of baited camera trap boxes can be used to collect clear and precise images (Littlewood et al., 2021). When comparing the sampling effort, eDNA metabarcoding exceeds camera traps for species detection. However, when examining the eDNA data, the differences within the two sampling sessions is stark with far more species detected in the second sampling session. In the first sampling session there is one unique species, *Lepus granatensis*. In the second sampling session there is six unique species detected, from four orders. *M. nivalis* and *G. servalina* from Carnivora, *Glis glis* from Rodentia, *Sorex quitan* and *Talpa quitania* from Eulipotyphla and *Dama dama* from the order Artiodactyl (Table 2.). It has been recommended that more effort or samples may been needed to accurately represent the overall biodiversity when using eDNA (Broadhurst et al., 2021; Lyet et al., 2021). These results highlight the increased accuracy of results when temporal sampling is used. We can argue that increased effort does not have to mean larger quantities, in size or amount, of samples in a sampling period but instead repeated sampling in short succession. Looking at the whole mammalian community, the combined results of both sampling periods have a higher number of species than either sampling session alone (Figure 6). Temporal seasonality changes in eDNA results have shown variation in species detection which can give useful insights into the biodiversity fluctuations for monitoring (Lacoursière-Roussel et al., 2018). However, short-term temporal resampling studies, like this, have shown greater representation in the overall recorded biodiversity (Beentjes et al., 2019). The first sampling session only identified 18 out of the 24 species compared to the second sampling session where 22 species were identified (table 2). Both sampling sessions were important to gain a better understanding of species community within the area, each sampling session identified species in all five orders, but a richer picture of the community was built with the data combined. The higher number of detections found in sampling session two could have also been due to the excess of rainfall after the first sampling session (R. Hannify, pers. comm.) which would have washed DNA from the surrounding area into the water system, explaining why so many carnivores were detected in the second sampling session (Lyet et al., 2021; Staley et al., 2021).

Species richness and abundance influence the shape of species accumulation curves (Thompson & Withers, 2003). Species that are infrequently recorded will cause long, upwards slope lines indicating more 'trapping' is required (Thompson & Withers, 2003). We can see this reflected in the overall mammalian eDNA data curve. More species were captured in the second sampling session, however more sampling could have led to more species identified which would have plateaued the curve (figure 6).

The second sampling session resulted in a greater amount of sequence data for the carnivores, which we can see by the larger bubbles which represents the amount of reads for carnivores found at each location (figure 8). Comparing the number of carnivore species detected by each sampling method we see more species were recorded by the camera traps (seven species or 'categories') than eDNA sampling (five species; Figure 4). Figure 10A shows the curve has plateaued for eDNA sampling but has not for camera traps indicating more sampling would need to be undertaken to capture all species, similarly when the methods are combined the curve still has not plateaued (figure 10B). Using species accumulation curves based on trapping (camera traps and eDNA sampling in this case) would be more accurate as there would be no observer bias in species recording (Colwell & Coddington, 1994). Thompson et al. (2003) found that species accumulation curves were useful to compare the estimated species richness between given sites; Broadhurst et al., (2021) looked at species accumulation curves split between orders within the sampling area. Here, species accumulation curves were used to compare richness estimates between two sampling methods. The results of this study show that even with the eDNA sampling supplementing 'traditional' methods more sampling would be required to capture all carnivores. Overall using both methods together would create a more rounded picture of the carnivores for the area.

4.2 Carnivore community presence and absence

With this small-scale study we have been able to show the validity of eDNA metabarcoding in the detection of carnivorous species in the north-eastern region of Spain. The large home ranges and behavioural ecology of carnivores is often reported as the reason for low eDNA reads within mammalian metabarcoding (Lyet et al.,2021; Sales et al., 2020a; Harper et al., 2019; Thompson et al., 2012). False negatives can occur due to the behavioural ecology of carnivorous species as they may not be active in the study area during the time of sampling. Species with large home ranges, such as carnivores, highlight the importance of temporal and spatial sampling as multiple sampling points may be required to detect rare/carnivorous species. eDNA degradation, seasonality affecting species behaviour and habitat variation all impacted the spatio-temporal reliability when sampling white-clawed crayfish (Troth et al., 2021). To target specific species additional sampling, both spatially and temporally, may be required to get reliable detections (Sales et al., 2020a). Lyet et al. (2021) have suggested sampling after heavy rainfall or after snow melts that cause more terrestrial DNA to enter the water, which theoretically would lead to a higher detection of carnivores based on successful soil-derived eDNA results (Leempoel et al., 2020). In this study, sampling session two was conducted after rain fall and the results showed a higher portion of mammals were detected than before the rainfall (Table 3).

Strictly terrestrial carnivores may only have contact with water for activities such as bathing and drinking which could explain why *G. servalina* was captured by the camera traps so frequently (19 out of the 25 sites) but was only identified in one eDNA sample (Harper et al., 2019; Lyet et al., 2021). Solitary species' DNA have been found to degrade at a higher rate to that of group living species in a laboratory setting, due to the amount of DNA secreted, which could also explain the low carnivore detection rates (Williams et al., 2018). Although, as stated by Williams et al. (2018), measurement of the environmental variability was not applied to the study so further research is needed to see if the

degradation of single species DNA is an important factor. DNA degradation rates will have important conservation implications if eDNA is the sole use for monitoring newly invasive or rare/solitary species.

Notably, *L. lutra* has the largest number of reads out of the carnivore species (Figure 6). This species has been difficult to detect in other mammalian eDNA studies (Broadhurst et al., 2021; Sales et al., 2020a; Harper et al., 2019) but has been much more regularly detected here. As this species lives a semi-aquatic lifestyle the assumption is that more DNA would be detected in the water. *L. lutra* reads have been noted to have weaker signals than other semi-aquatic mammals (Harper et al., 2019). Here *L. lutra* visually has the largest proportion of reads of any carnivore, semi-aquatic or terrestrial (Figure 6). Although some of *L. lutra*'s behaviour may not lead to DNA shed in the water, as territory marking and spraints are often done outside the water (Mason & Macdonald, 1986), there was evidence during the eDNA sampling of crayfish carcasses around the water edge which would imply that *L. lutra* was hunting and eating in the water (Barrientos et al., 2014). *L. lutra* was detected in both sampling sessions which could be due to seasonality and placement of the individuals at the time of sampling. However as biotic and abiotic factors were not monitored within this study eDNA results can not accurately be interpreted into abundance (Tillotson et al., 2018). However, this study demonstrates that the application of eDNA for monitoring otters holds more promise than previous studies have shown (Broadhurst et al., 2021; Sales et al., 2020a; Harper et al., 2019).

V. vulpes, a strictly terrestrial carnivore, is found in relatively high abundance consistently throughout the sampling area (Figure 6). Lyet et al. (2021) found that omnivorous predators were more likely to be detected than strict carnivores during the time of sampling. We found this to be true when comparing the omnivorous predators (*V. vulpes, M. meles,* and *M. lutreola*) to strict carnivores (*M. nivallis*). *G. servalina* (omnivore) and *L. lutra* (carnivore) however, did not fit with this trend.

M. meles was detected by eDNA (seven out of 25 sites) but was not detected by the camera traps once. These results reflect the findings from Broadhurst et al. (2021) where *M. meles* was detected regularly (seven sites out of 25 also). Although *M. meles* can swim, they often chose not to do so and

this may mean that they do not come in close contact with the water often (Sleeman et al., 2009). This could explain its absence on the camera traps. Despite their preference to avoid water, DNA was excreted by or carried to the rivers, which could account for the higher number of reads recorded in the second sampling session after heavy rainfall (Figure 6; Lyet et al., 2021). For the original study the VWT positioned 50 camera traps around water ways as their main aim was to capture semi-aquatic species (*M. lutreola* and *N. vison*). Despite using two cameras at each site the placement of the study species can influence other species detection, if the cameras are low and facing the water there is a chance that some terrestrial species will not be captured, which could be true for *M. meles* (Leempoel et al., 2020; Sales et al., 2020a; Hofmeester et al., 2019).

One bias for high carnivore detection rates we see within this study, which is not replicated in other mammalian eDNA studies, is the use of bait. As the original study with the VWT was actively trying to capture mink they had all 50 camera traps baited with sardines to encourage sightings. This may explain why so many carnivores were detected with eDNA as they would be attracted to the water's edge by the bait. However, this would not explain the absence of some species on the camera traps. As we were not testing to see if bait attracted any of the carnivores preferentially, we cannot determine if this influenced the eDNA results. *M. lutreola* was detected at 4 out of the 5 UTM squares with either camera trap or eDNA, with no detections recorded at UTM 2 (sites 6 – 10; Figure 9). Five out of the 16 positive detections were recorded by both camera traps and eDNA, at sites 2, 18, 19, 22 and 24 and only eDNA detected *M. lutreola* at UTM 3 (sites 11 – 15; Figure 9). As the original study with the VWT was to see if eDNA was comparable to camera traps these results show that eDNA can perform well in comparison to the camera traps for *M. lutreola* detections.

One of the advantages of using eDNA metabarcoding for species identification is the accuracy which some traditional methods cannot achieve (Leempoel et al., 2020). In this study, five carnivorous species were identified with a >0.98 sequence match (one species at 0.95) with one of these species not identified by the camera traps. Camera traps recorded seven carnivorous 'groups' of animals, with only five of these identified to species level and two to family name (*Martes* and *Mustela*, common

name marten and weasel) (Figure 4). With species declines being one of the biggest concerns of this era, accurate species identification is of paramount importance. eDNA could provide early detection (or absence) of endangered species which could impact how plans will be made for the conservation of species.

N. vison was not recorded by eDNA sampling and was caught on only one occasion by the camera traps. The species was not detected during concurrent live trapping either (M. Podra, pers. comm). Broadhurst et al. (2021) has presented similar results where *N. vison* was detected once via eDNA at one site in an area that was known to be at the edge of a *N. vison* eradication zone. Our results could explain why there was no reads from this species if it was scarcely in the area. Browett et al. (2020) highlighted the potential of using eDNA as an early detector for invasive species and both the results from this study and Broadhurst et al. (2021) could have large conservation implications as positive and negative detections of an invasive species was made. However, as *N. vison* was not amplified in this study, specific research into invasive species may want to use a species-specific qPCR or digital PCR (ddPCR) assay to avoid any 'species masking' which may occur with low reads (Harper et al., 2018). Another explanation could be inaccurate identification from the camera traps. One of the most common errors in data collection from most traditional methods is the misidentification of a species, especially when there are two species that look extremely similar, such as *N. vison* and *M. lutreola*. *N. vison* and *M. lutreola* have few distinguishing features to tell them apart, *N. vison* is slightly larger with a white marking on the chin whilst *M. lutreola* has the white mark on the chin which extends up above the mouth, to get an accurate identification of the mink species a clear image of the face needs to be captured. This process of identification can, understandably, lead to misidentification with blurry images or body images, when identifying mink species within this study an 'unsure mink' category was made for the images where an identification could not be made. This method of identification can lead to both false positive and negatives. Research into *L. lutra* and *N. vison* cohabitation has found that *N. visons* has a generalist and opportunistic diet has meant that they can share similar niches. *L. lutras* prowess in the water means their diet can stay mainly aquatic whilst *N. vison* can hunt terrestrial

prey as well. *N. vison* may not have been detected using eDNA due to its current hunting habits and number of otters (Harper et al., 2020). *N. vison* has been reported to shift their food preference to terrestrial prey during the winter months which could coincide with rising otter densities (Bonesi and Macdonald, 2004). As the sampling for this study was conducted in November, seasonality could be important when trying to detect species such as *N. vison*.

Lyet et al. (2021) recommend filtering a larger volume of water to capture the carnivore community presence/absence more accurately, whilst noting that it would however increase both the cost and manpower needed. Lyet et al. (2021) hypothesised that a larger volume of filtered water is necessary to capture all species in the community accurately, however both this study and the one conducted by Broadhurst et al. (2021) only filtered 500ml per replicate and were still able to collect abundant data for the mammalian community, with both studies able to identify five orders compared to Lyet et al. (2021) with four orders. The functionality of filtering larger amounts of water will start to push eDNA into exclusivity, with the increased costs and challenges of filtering larger amounts requiring more specialist equipment. This means that it becomes more difficult to conduct eDNA studies that are more accessible (for example to citizen scientists). This would also alter the standardisation for eDNA studies if it was recommended to filter larger quantities, but some researchers are only able to filter small quantities. From the results of this study, we were able to gather data on a wide variety of species from five orders using only 500ml of filtered water per replicate (2.5 L per sampling site at each visit). For future studies an approach with more emphasis on temporal sampling to capture the carnivorous community would be recommended.

More emphasis on seasonality, species behaviour ecology and diet may need to be considered when trying to detect carnivores as these have been highlighted as potential causes of low/no reads and/or detections within eDNA mammalian studies (Sales et al., 2019; Harper et al., 2019; Leempoel et al., 2020; Broadhurst et al., 2021; Lyet et al., 2021). This study showed the importance of short scale

temporal studies, especially within carnivore detection as only four species were detected in the first sampling session compared to the second sampling session where six were detected (Table 2).

4.3 Carnivore occupancy modelling

When comparing the two-sampling methods, occupancy modelling can provide an unbiased method of taking imperfect data with variation to account for species occurrence (MacKenzie et al., 2002). Direct comparisons can be made from the two sampling methods in this study of multiple species simultaneously (Sales et al., 2020a; Broadhurst et al., 2021). For the comparisons *M. meles* and *N. vison* were not included as they were not detected with both camera traps and eDNA sampling. The probability of detecting the remaining carnivores (*V. vulpes*, *M. lutreola*, *L. lutra*, *M. nivallis* and *G. servalina*) was higher overall for the camera traps (Table 4). The largest difference in detection probabilities between the two methods occurred in the detection of *G. servalina* and *M. lutreola.* However, when considering the confidence intervals with the two methods there is overlap in the results for *M. lutreola* but not for *G. servalina,* this is due to only being detected in one eDNA replicate out of 250 but having multiple captures on the camera traps (Table 4). Similar detection probabilities for camera traps and eDNA were found in the remaining carnivores, again with large overlap in the confidence intervals. Sales et al. (2020a) found the eDNA outperformed camera traps in detection probabilities in three focal species, however these were not carnivorous species, in their small-scale study. Broadhurst et al. (2021) found that detection probabilities for three comparable carnivorous species had similar results in two of the species, *V. vulpes* and *L. lutra*, however there was a higher detection probability found for *M. nivallis*. Broadhurst et al. (2021) found that eDNA detection probabilities for carnivores ranged from 0.01 to 0.80 whereas the ranges for this study were considerably less, 0.01 – 0.27 (Table 4). Results were generally comparable however for most, with one species (*N. vision*) skewing the results from Broadhurst et al. (2021).

Per each UTM square, both monitoring methods would be required to capture all eight carnivore species. UTM 2 did not record any of the carnivorous species with both camera traps and eDNA (Figure 9). Removing camera trap data would mean both *N. vison* and *Martes* would not be identified, similarly relying on only camera trap data would mean *M. meles* would have been missed. A recommendation of eDNA sampling to supplement camera trap data for monitoring carnivore species would be made at this stage as the use of only one of the monitoring methods would mean less species detections overall (Sales et al., 2020a; Abrams et al., 2019).

Large home ranges are often assumed to be the main reason for lack of carnivore eDNA detections and recommendations to sample larger areas are standard, however we have shown how a multi-sampling approach that incorporates different environmental conditions can accumulate results for carnivore detections. Although camera trap detection probabilities were overall higher than eDNA for carnivores, we would recommend a combination of methods to gain better overall data on the community. As this study was focusing on a semi-aquatic carnivore, the camera traps were positioned to reflect this thus failing to account for the heterogeneous landscape and capturing other terrestrial species. As this was not the aim of this study, future studies looking into carnivore eDNA will want to place cameras in different habitats to account for the different lifestyle of individual carnivore species.

5. Conclusion and future suggestions

This study demonstrates the power of eDNA when sampling semi-aquatic and terrestrial species and shows how more precise species identification can be made in comparison to traditional methods (Table 2 and 3). The success in detecting carnivores within this study demonstrates how the multi-sampling approach is beneficial when attempting to detect large-ranging, solitary species (Ficetola et al., 2008). There are recommendations to sample larger quantities of water with the aim of detecting more species (Lyet et al., 2021), however we effectively detected multiple mammals using smaller quantities per site. We recommend rather than sampling larger quantities of water, numerous sampling sessions could be carried out. Frequent sampling will accurately account for abiotic factors that may be affecting detection, as well as possibly allowing for monitoring of population fluctuations. The standardisation of eDNA methodology and the use of citizen science would allow for long-term biodiversity monitoring of mammals and those harder to detect groups, such as carnivores. Standardisation of eDNA sampling will make for a more robust and comparable monitoring technique (Harper et al., 2018). eDNA outperformed camera traps on the mammalian monitoring overall. Although camera traps outperformed eDNA when specifically looking at carnivore detections using the combined methods would see the most reliable results for mammalian species monitoring. An important factor to consider in future eDNA studies is the lack of accurate sequences available in public databases (Axtner et al., 2019). Although the threshold for this study for accurate identification was >0.98 manual identification found four species below this threshold at 0.93, 0.94, 0.95 and 0.97. Leempoel et al. (2020) found 59% of mammals were missing from the 12S database with 33% of these having no genus level sister species. 62% of missing carnivore species also had no sister species which could explain why there is an absence in carnivore detections within other mammalian eDNA studies. The incompleteness of the database means there is a high possibility of false species absences noted with other eDNA studies (Jackman et al., 2021; Sales et al., 2020b). Building a global, comprehensive DNA database will drastically improve the quality of future eDNA studies. Projects such as ‘DNAmark’,

a national DNA reference database for species found in Denmark, is contributing towards a global database which will enrich future DNA biodiversity monitoring (Margaryan et al., 2020).

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