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# **Vertebrate consumption by raccoon in two Walloon forests: insights from DNA metabarcoding on faecal samples**

Nick Dillen, Charlotte Van Driessche, Teun Everts, Sabrina Neyrinck,  
David Halfmaerten, Berdien De Beer, Hanne Danneels & Rein Brys

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## Samenvatting

De wasbeer (*Procyon lotor*) is een invasieve uitheemse soort die snel oprukt in België en Europa. Om de impact van deze soort op de lokale biodiversiteit te kunnen inschatten is een beter inzicht in zijn dieet noodzakelijk. In deze studie analyseerden we het dieet van 107 wasberen op basis van hun uitwerpselen, verzameld over een periode van twee jaar in twee bossen in Wallonië.

Aan de hand van een gestandaardiseerde *environmental* DNA metabarcoding pipeline brachten we de gewervelde fauna in hun uitwerpselen in kaart. De resultaten bevestigen dat de wasbeer een omnivoor is met een erg gevarieerd dieet, waaronder amfibieën, vissen, vogels en kleine zoogdieren. Naast algemene soorten, zoals bruine kikker en gewone pad, vonden we ook kwetsbare en zeldzame soorten, zoals vuursalamander, kamsalamander, rivier/beekprik en wespendif.

Dit onderzoek toont aan dat de wasbeer niet kieskeurig is wat betreft zijn dieet, en een brede ecologische niche inneemt. Vervolgonderzoek is nodig om de exacte kwantitatieve impact op de inheemse fauna en ecosystemen vast te stellen, en de mogelijke overlast voor landbouw en stedelijke gebieden in kaart te brengen.

## Summary

The raccoon (*Procyon lotor*) is an invasive alien species that is rapidly expanding in Belgium and Europe. To assess the impact of this species on local biodiversity, a better understanding of its diet is necessary. In this study, we analyzed the diet of 107 raccoons based on their scats, collected over a two-year period in two forests in Wallonia, Belgium.

Using a standardized environmental DNA metabarcoding pipeline, we mapped the vertebrate fauna in their scats. The results confirm that the raccoon is an omnivore with a highly varied diet, including amphibians, fish, birds, and small mammals. In addition to common species, such as the common frog and common toad, vulnerable and rare species were detected, such as the fire salamander, great crested newt, river/brook lamprey and European honey buzzard.

This study demonstrates that the raccoon is an opportunistic feeder occupying a broad ecological niche. Further research is needed to determine the exact quantitative impact on native fauna and ecosystems, as well as to map its potential effects on agriculture and urban areas.

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## Aanbevelingen voor beheer en/of beleid

Deze studie is uitgevoerd in een redelijk beperkt gebied, nl. twee boscomplexen in Wallonië, toch biedt ze enkele concrete inzichten voor het toekomstige beheer van wasbeer in Vlaanderen.

- De resultaten bevestigen de algemene bezorgdheid over de potentieel negatieve impact van wasbeer op de lokale biodiversiteit. Het dier blijkt inderdaad een generalistische omnivoor die zijn dieet zowel op land, in het water, of zelfs hoog in de bomen vindt, en het zowel uit inheems als uitheemse, algemene en zeldzame soorten samenstelt. Nagenoeg alle soorten die in deze dieetstudie naar voor kwamen, komen ook voor in Vlaanderen. We kunnen verwachten dat deze soorten eveneens op het menu zullen staan van de oprukkende wasbeer.
- De aanwezigheid van inheemse gewervelde fauna in het dieet van wasbeer bevestigt de risicobeoordelingen van Europese experts dat wasbeer een reële dreiging voor een tal van Europese soorten met zich meebrengt, waardoor deze soort terecht is opgenomen als zorgwekkend voor de Europese Unie in EU Reguleratie 1143/2014. De verplichting die Europa oplegt om in te grijpen, al is het maar om de schade te beperken, is m.a.w. noodzakelijk.
- Een dieetstudie kan inzichten geven omtrent de potentiële impact van een soort op de lokale fauna. Het is echter niet mogelijk om hiermee ook een effectieve impact te kwantificeren. Op basis van deze resultaten is het dus moeilijk in te schatten in welke mate de predatiedruk van wasberen een impact zal hebben op de populaties van de geconsumeerde soorten. Wel kan aangenomen worden dat bepaalde inheemse populaties een extra druk zullen ondervinden. Omdat verwacht kan worden dat de predatiedruk stijgt met een toenemende grootte van de wasbeer populatie, kan de grootte van de impact op lange termijn beperkt worden door wasberen te bestrijden.
- Vlaanderen staat voor een cruciale keuze. Hoewel meer kennis nodig is om de exacte impact van de wasbeer te voorspellen, mag dit een preventieve aanpak niet in de weg staan. Integendeel, de vraag is of we afwachten tot de mogelijke schade aan onze inheemse soorten en ecosystemen zich effectief manifesteert, dan wel tijdig ingrijpen om te voorkomen dat kwetsbare inheemse soorten nog verder onder druk komen te staan.

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## Recommendations for management and policy

While the study area is limited to two areas in the Walloon region in Belgium, the results still allow recommendations regarding the future management of the raccoon in Flanders.

- The results confirm the general concern regarding a potential negative impact of raccoons on local biodiversity. The animal is a generalistic omnivore that finds its food on land, in water and even high up in the trees, and consumes local, alien, common and rare species alike. Virtually all species presented in this diet analysis occur in Flanders as well.
- The detection of local vertebrate fauna in the raccoon's diet supports the risk analyses by EU experts, who listed the raccoon as a serious threat to European biodiversity in EU regulation 1143/2014. The obligations imposed by the EU to contain and manage listed invasive alien species are necessary, even if only to contain part of the damages.
- While a diet study can provide multiple insights into the local fauna, it can not quantify the effective impact. Based on the presented results it is not possible to prove any real effect of raccoon predation on native prey populations. However, it is expected that certain native (and possibly vulnerable) species will experience an increase in predation pressure that scales with raccoon population density. An active and direct management can limit or control the size of the impact in the long term.
- Flanders is at a crossroads. Even if more knowledge is needed to predict the exact impact of the advancing raccoon, preventive management strategies should not be dismissed. On the contrary, waiting for a conclusive decline in our native species populations and ecosystems before taking action might lead to irreversible effects.

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# 1 Introduction

Invasive alien species are considered one of the major threats to biodiversity and represent a prime challenge to the conservation of natural ecosystems (Haubrock *et al.*, 2025). Originating from North and Central America, the raccoon (*Procyon lotor*) was introduced to Germany in the late 1920's. In the 1970's the wild raccoon population in Germany expanded to neighbouring countries, including Belgium. Since the 1990s, it has grown at exponential rates (Louppe *et al.*, 2019; Salgado, 2018), claiming its status as an invasive alien species throughout Europe (Figure 1). Given its large potential impact on native species, the raccoon is listed as an alien species of European Union (EU) concern, under EU Regulation 1143/2014.

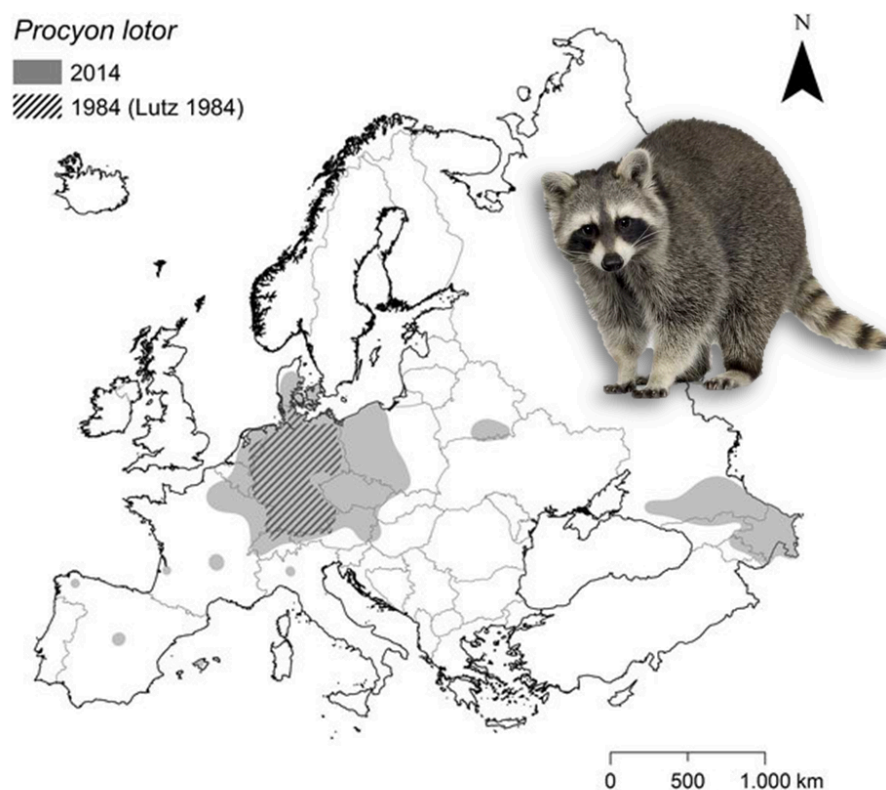


**Figure 1.** Distribution of the northern raccoon (*Procyon lotor*) in Europe. Both records of established (reproduction) and invasive (range expansion) populations were used to build the species distribution map. The striped area represents the raccoon-invaded range in central Europe estimated by Lutz (1984). Figure is adapted from Salgado, 2018.

In Belgium, the species has rapidly expanded since 2007 and is now established across large parts of the Walloon Region (Figure 2). The Ardennes and adjacent massifs currently represent core areas of high raccoon density.

**Figure 2.** Expansion of raccoon (*Procyon lotor*) occurrence across Wallonian municipalities, showing a steady temporal increase from 2007 through 2025.



This study was initiated by DEMNA (Département de l'Étude du Milieu Naturel et Agricole, Gembloux), who collected faecal samples during ongoing management campaigns in order to control the raccoon population(s) in the Walloon Region (Figure 4). The objective of this study is to investigate the vertebrate diet of raccoons captured in Walloon forests in the Saint-Michel-Freyr massif, a species-rich forested area of conservation importance in southern Belgium (Figure 5). Using DNA metabarcoding we aim to characterise the vertebrate prey composition of raccoon diets to assess the contribution of native species to their food intake. These results directly support the impact assessment of this non-native invasive species required under the EU Regulation No 1143/2014 on invasive alien species of Union concern, by providing a molecularly verified baseline of raccoon predation on co-occurring vertebrate fauna in this region.

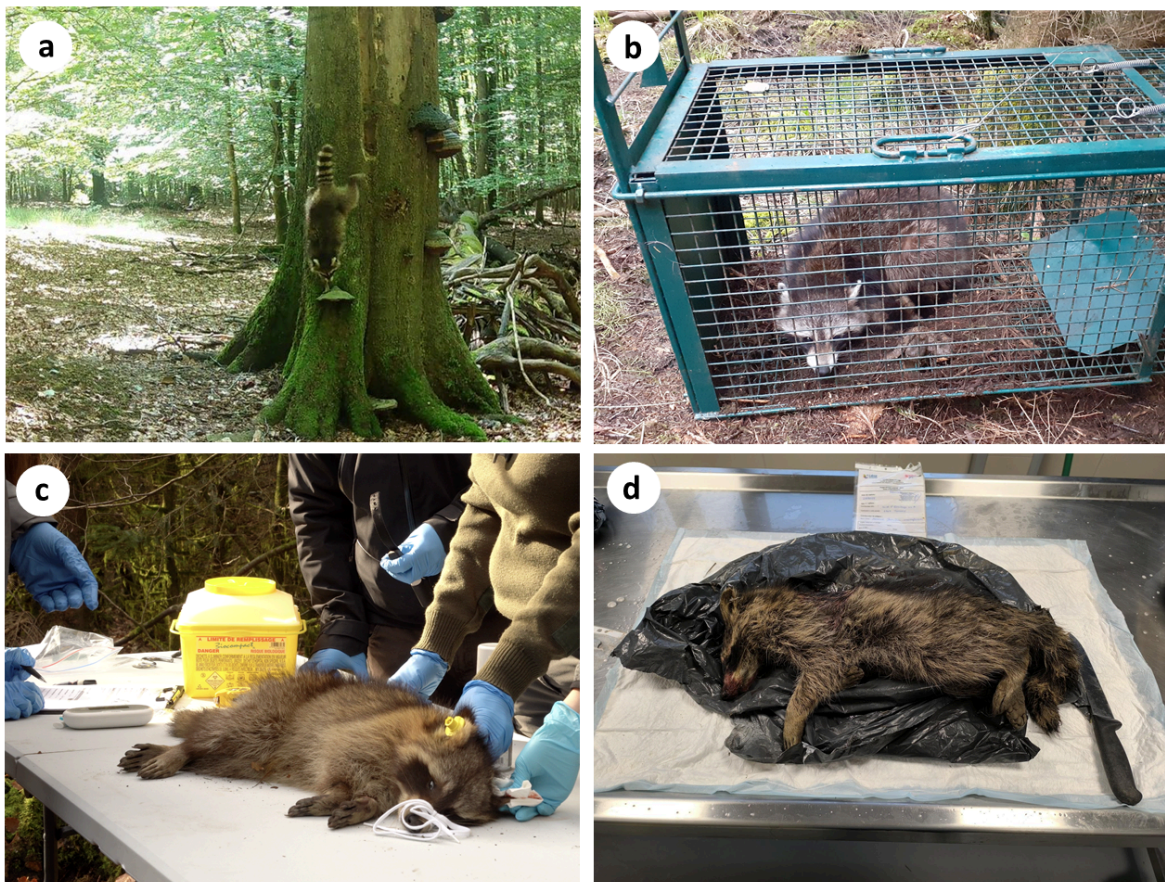


**Figure 4.** Spring 2024 field campaign, involving (a) monitoring raccoon presence using camera traps, (b) capturing raccoons, (c) anesthetizing and measuring raccoons, and (d) performing autopsies on raccoons, during which faecal samples were collected and stored.



## 2.2 Sample collection and transport

In 2024 and 2025, raccoons were captured as part of ongoing management campaigns conducted by the Département de l'Étude du Milieu Naturel et Agricole (DEMNA, Gembloux). Cages with bait were deployed in the southernmost tip of Belgium (Figure 4b, 5). Although the exact composition of the bait was unknown, it consisted partly of dog food and partly of a mixture of marine fish. Faecal samples were collected from 107 captured raccoons (Table 1), preserved in ethanol and transported to the laboratories of the Research Institute for Nature and Forest (INBO) in Geraardsbergen for analysis. Prior to delivery, DEMNA subdivided each faecal sample into duplicate subsamples to enable a blind comparison of parallel analyses conducted independently between both INBO and the Laboratoire de génétique de la conservation (GeCoLAB, Liège).

**Table 1.** Overview of the 107 raccoon faecal samples grouped per sampling campaign.

| Sampling time | Number of samples |
|---------------|-------------------|
| Spring 2024   | 23                |
| Summer 2024   | 44                |
| Spring 2025   | 40                |
| <b>Total</b>  | 107               |

Dietary analyses were performed using a standardised, high-quality environmental DNA (eDNA) metabarcoding workflow routinely implemented at INBO for vertebrate community composition assessment (Brys *et al.*, 2020) (Figure 6). This workflow includes three main steps: DNA extraction, eDNA metabarcoding, and bioinformatic analyses, each described in the subsections below.

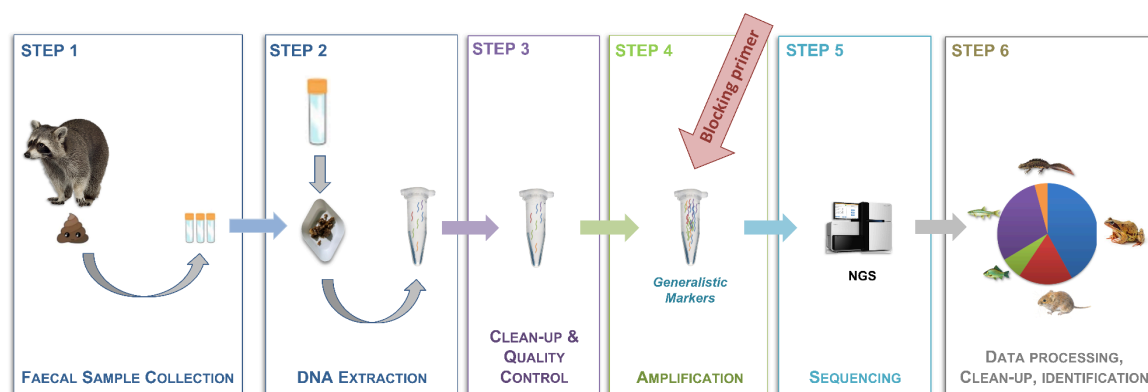


**Figure 6.** Schematic overview of the eDNA metabarcoding workflow developed and routinely implemented at INBO for vertebrate diet analysis assessments.

### 2.3 Sample preparation and laboratory analyses

A substantial proportion of the samples had been preserved in ethanol containing denatonium benzoate and methyl ethyl ketone (MEK), both known to inhibit DNA extraction and negatively affect metabarcoding performance (Prins *et al.*, 2024). To mitigate these effects, each faecal sample was resuspended in 1.5 L of ultrapure water and homogenised thoroughly. After settling for 10 minutes, 1 L of the upper, least turbid fraction was filtered through a Sterlitech eDNA capsule (0.8 µm PES membrane). Finally, eDNA was extracted from the filters using the Qiagen Blood & Tissue extraction kit following the INBO aquatic eDNA extraction protocol as described in Brys *et al.*, 2020 and Everts *et al.*, 2022.

A series of control steps were incorporated to assess the quality and the efficiency of the eDNA workflow:

- **Internal Positive Extraction Control (IPEC):** Exogenous DNA from a species known to be absent in the study area (in this case, Dengue virus DNA) (Brys *et al.*, 2020) was added to each sample. This control guards against false-negative detections (i.e. incorrectly concluding that a species' DNA is absent from the faecal sample) caused by possible issues during DNA extraction.
- **Positive PCR Control:** DNA extracts from a separate project with a highly distinct and well-characterised vertebrate community composition were simultaneously analysed on the same sequencing run. This control guards against false-negative results due to potential errors during PCR amplification or sequencing.
- **Negative PCR Controls (NTC):** DNA-free laboratory blanks treated identically to the eDNA samples were incorporated at both the DNA extraction stage and during PCR amplification. These controls check for potential contamination with extraneous DNA during the workflow, guarding against false-positive detections (i.e. incorrectly concluding that a species' DNA is present in the faecal sample).

## 2.4 Blocking primer optimisation

A recurrent issue when analysing faecal samples is the overabundance of host DNA (raccoon in this case), limiting the detection of non-host species signals. To increase the effective read depth of prey-derived eDNA from raccoon faecal samples a host-specific blocking oligonucleotide was implemented during PCR, based on the RacBlk nucleotide developed by Woo *et al.* (2022). This 27bp long blocking oligonucleotide is reported to selectively inhibit amplification of the 12S rRNA of the closely related raccoon dog (*Nyctereutes procyonoides*) without significantly altering vertebrate prey community composition (Woo *et al.*, 2022).

However, DNA alignment of the RacBlk nucleotide with that of the common raccoon revealed a 1 basepair mismatch in the binding region. Because of this mismatch RacBlk is expected to suboptimally inhibit DNA amplification of the host in this study. To ensure optimal theoretical

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inhibition of host DNA amplification, the RacBlk nucleotide sequence was adapted to exactly match that of the raccoon host DNA (Table 2), and named NARacBlk.

**Table 2.** Comparison of blocking oligonucleotides. A single base-pair substitution at nucleotide position 13 (5'→3'; A→G) was introduced relative to the original blocking primer designed for raccoon dog (*Nyctereutes procyonoides*), to ensure an exact match with the raccoon (*Procyon lotor*) host sequence.

| Target                          | Name     | Nucleotide sequence (5' -> 3')         |
|---------------------------------|----------|----------------------------------------|
| <i>Nyctereutes procyonoides</i> | RacBlk   | CTCTAGAGGGAT-A-TAAAGCACCGCCAA          |
| <i>Procyon lotor</i>            | NARacBlk | CTCTAGAGGGAT- <b>G</b> -TAAAGCACCGCCAA |

The original RacBlk was reported to also suppress PCR reaction of taxa with a very similar or identical target region of the 12S rRNA gene to the raccoon dog, namely other species of *Canidae*, including domestic dog, red fox and grey wolf. To investigate the effect of the 1bp change in NARacBlk, a sequence alignment was made including mammals likely to occur in the study area. The NARacBlk binding sequence is, next to raccoon, identical in species like horse (*Equus f. caballus*), house cat (*Felis catus*), and *Mustelidae* species like European otter (*Lutra lutra*), European badger (*Meles Meles*), martens (*Martes*) and weasels (*Mustelae*). If these species are part of the raccoon diet in the study area, amplification of their target DNA will also be blocked, resulting in an underestimated or completely absent signal (false negative) of these species.

The effect of NARacBlk was empirically evaluated on a subset of 12 samples selected from the three sampling campaigns. A single sample did not deliver any sequencing data and was excluded from further analysis. For the remaining 11 samples the effective read depth for non-raccoon eDNA in blocked and unblocked (control) PCR reactions was compared. Results revealed a strong dominance of raccoon host DNA in the sequencing output when no blocking primer was used, possibly limiting detection of non-raccoon taxa including amphibians, birds, fish, and other (non-raccoon) mammals (Figure 7a) due to insufficient sequencing depth. In the blocked reaction, the relative read contribution of non-raccoon taxa increased significantly compared to raccoon host DNA (Figure 7b), although the latter could not be completely blocked. Based on these results, we decided to use the NARacBlk blocking nucleotide in the routine workflow and applied it to the full set of 107 samples.

[illegible]



## 2.5 Metabarcoding workflow

A standardised metabarcoding protocol was used to extract and amplify the target 12S fragment in all samples, incorporating 3 technical replicates per sample and the aforementioned control steps (Brys *et al.*, 2020; Van Driessche *et al.*, 2024).

This approach uses the general vertebrate primers (12S\_F1, 5'-ACTGGGATTAGATACCCC-3'; 12S\_R1, 5'-TAGAACAGGCTCCTCTAG-3') (Riaz *et al.*, 2011) to target an approximately 85-117bp (excluding primers) fragment located on the mitochondrial 12S locus. This barcode enables accurate detection and identification of all native West-European mammals, amphibians and freshwater fish. INBO has created a curated reference database for the 12S amplicon (Halfmaerten *et al.*, 2023), fine tuned to allow species-level identifications for these taxa in Belgium.

While in this study data on bird detections are also reported, it has to be noted that neither the amplicon nor the reference database were developed to resolve bird taxonomy in detail. In most cases only higher level taxonomies can be reported and missing taxa are to be expected. Even though bird detections are likely incomplete, they can give a general indication on the contribution of poultry to the raccoon's diet.

## 2.6 Bioinformatic analysis and data preparation

After DNA amplification, library preparation, and quality control, all 107 samples were sequenced on an Illumina NovaSeq platform. Sequence data were filtered and cleaned according to a standardised bioinformatic pipeline (Brys *et al.*, 2020; Van Driessche *et al.*, 2024). Briefly, raw paired-end reads were demultiplexed, merged, trimmed of primers and filtered on basic sequence quality metrics. High quality reads were subsequently denoised into zero-radius operational taxonomic units (zOTUS) and assigned taxonomy using a custom, in-house, and carefully curated 12S reference database (Halfmaerten *et al.*, 2023). zOTU sequences matching a reference sequence with 100% similarity were retained in the downstream analysis. zOTUs that did not match completely with a reference sequence were, in all but 2 cases, excluded from further analysis as these are expected to be PCR or sequencing artefacts. The two excepting cases are a *Pernis apivorus* zOTU and an additional *Apodemus* zOTU that, despite having a single basepair mismatch with the reference sequences, are very likely to belong to a real biological signal and thus retained.

Based on the sequencing results of the positive and negative amplification controls, samples with fewer than 15,000 total reads and zOTUs with fewer than 2,000 total reads across the entire sequencing run were excluded from further analysis to ensure a high likelihood of true-positive detections. After division of the samples into subprojects, and further quality filtering, 100 out of 107 faecal samples retained enough reads to pass this criterion. Two samples could not be assigned to a specific raccoon individual; the sample labelled “Y.” was a faecal sample found next to a cage, while the sample labelled “?” did not receive a label during sample collection.

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IPEC control amplification confirmed successful DNA extraction and the absence of PCR inhibition. Positive PCR controls yielded the expected vertebrate community composition, confirming reliable PCR amplification and sequencing performance. No contamination was detected in the negative PCR controls (NTCs).

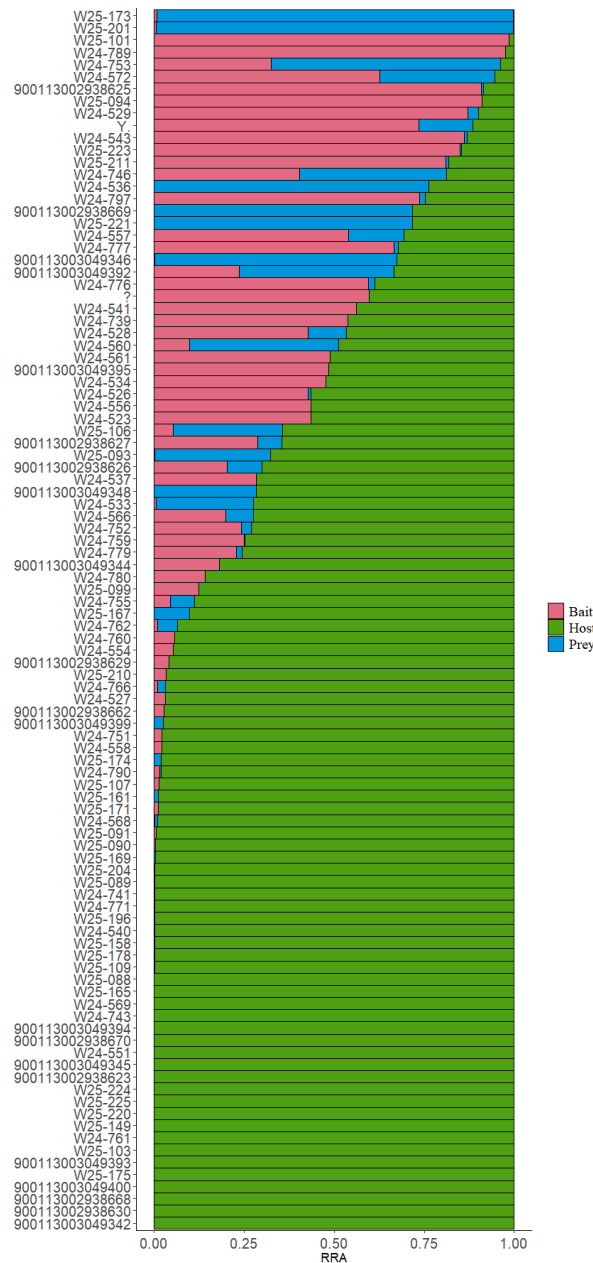


**Figure 8.** Relative Read Abundance (RRA) per functional category (host, bait and prey) across the 100 retained raccoon faecal samples, ranked by increasing proportion of host RRA. Each bar represents one sample, with green indicating raccoon host DNA, red indicating bait-derived taxa, and blue indicating prey taxa.

In total, 197 million high-quality reads were generated across the 100 retained samples, yielding 47 detected taxa. Raccoon host DNA dominated the sequencing output, comprising 74.5% of all reads (146 million reads). The remaining 50 million reads (25.5% of the total dataset) were distributed across 46 non-raccoon taxa. Of these, 16 taxa, amounting to 28 million reads (14.4% of the total dataset), were classified as bait-derived. The remaining 30 taxa accounted for 22 million reads (11.1% of the total dataset) and were classified as part of the raccoon's diet, most likely as prey.

Bar chart showing the prevalence of 50 species of ticks. The y-axis represents Prevalence from 0 to 100. The x-axis lists species names. Bars are color-coded: Bait (pink), Host (green), and Prey (blue).

| Species                                            | Prevalence (%) | Category |
|----------------------------------------------------|----------------|----------|
| <i>Procyon lotor</i>                               | 100            | Host     |
| <i>Gallus gallus</i>                               | 60             | Bait     |
| <i>Sus scrofa</i>                                  | 58             | Bait     |
| <i>Sardina pilchardus</i>                          | 35             | Bait     |
| <i>Bos taurus</i>                                  | 35             | Bait     |
| <i>Meleagris gallopavo</i>                         | 25             | Bait     |
| <i>Scomber japonicus</i>                           | 23             | Bait     |
| <i>Rana temporaria</i>                             | 22             | Prey     |
| <i>Thynnus alalunga</i>                            | 15             | Bait     |
| <i>Engraulis encrasicolus</i>                      | 14             | Bait     |
| <i>Scombrinae</i>                                  | 13             | Bait     |
| <i>Cervus elaphus</i>                              | 12             | Bait     |
| <i>Anatinae</i>                                    | 8              | Prey     |
| <i>Trachurus trachurus</i>                         | 8              | Bait     |
| <i>Bufo bufo</i>                                   | 7              | Prey     |
| <i>Apodemus sylvaticus</i> / <i>A. flavicollis</i> | 6              | Bait     |
| <i>Spondyliosoma cantharus</i>                     | 5              | Bait     |
| <i>Lissotriton helveticus</i>                      | 5              | Prey     |
| <i>Gallinula chloropus</i>                         | 5              | Prey     |
| <i>Columba</i>                                     | 4              | Prey     |
| <i>Ichthyosaura alpestris</i>                      | 4              | Prey     |
| <i>Lissotriton vulgaris</i>                        | 4              | Prey     |
| <i>Salamandrina salamandrina</i>                   | 4              | Prey     |
| <i>Numida meleagris</i>                            | 4              | Bait     |
| <i>Turdus</i>                                      | 3              | Prey     |
| <i>Ovis aries</i>                                  | 3              | Prey     |
| <i>Pelophylax sp.</i>                              | 3              | Prey     |
| <i>Triturus cristatus</i>                          | 3              | Prey     |
| <i>Carassius sp.</i>                               | 3              | Prey     |
| <i>Cottus rheneanus</i>                            | 2              | Prey     |
| <i>Pseudorasbora parva</i>                         | 2              | Prey     |
| <i>Cyprinus carpio</i>                             | 2              | Prey     |
| <i>Lepomis gibbosus</i>                            | 2              | Prey     |
| <i>Argyrosomus regius</i>                          | 2              | Bait     |
| <i>Salmo salar</i>                                 | 1              | Prey     |
| <i>Pernis apivorus</i>                             | 1              | Prey     |
| <i>Lampetra fluviatilis</i>                        | 1              | Prey     |
| <i>Tinca tinca</i>                                 | 1              | Prey     |
| <i>Rutilus rutilus</i>                             | 1              | Prey     |
| <i>Meles meles</i>                                 | 1              | Prey     |
| <i>Gasterosteus aculeatus</i>                      | 1              | Prey     |
| <i>Fulica atra</i>                                 | 1              | Prey     |
| <i>Myodes glareolus</i>                            | 1              | Prey     |
| <i>Fringilla</i>                                   | 1              | Prey     |
| <i>Garrulus glandarius</i>                         | 1              | Prey     |
| <i>Micromesistius poutassou</i>                    | 1              | Prey     |
| <i>Passeriformes</i>                               | 1              | Prey     |

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Raccoon reads were present in all 100 samples (Figure 9 and 10). Bait taxa showed consistently higher prevalence across samples than prey taxa, which is consistent with the use of similar bait in different locations (Figure 9). Among bait-derived taxa, farm animals like *Gallus Gallus* (chicken), *Sus scrofa* (pig), *Bos taurus* (cow) and *Meleagris gallopavo* (turkey), and marine fish species such as *Sardina pilchardus* (pilchard) and *Scomber japonicus* (mackerel) showed the highest prevalence, consistent with the expected used bait types, i.e. commercial dog food and/or canned fish.

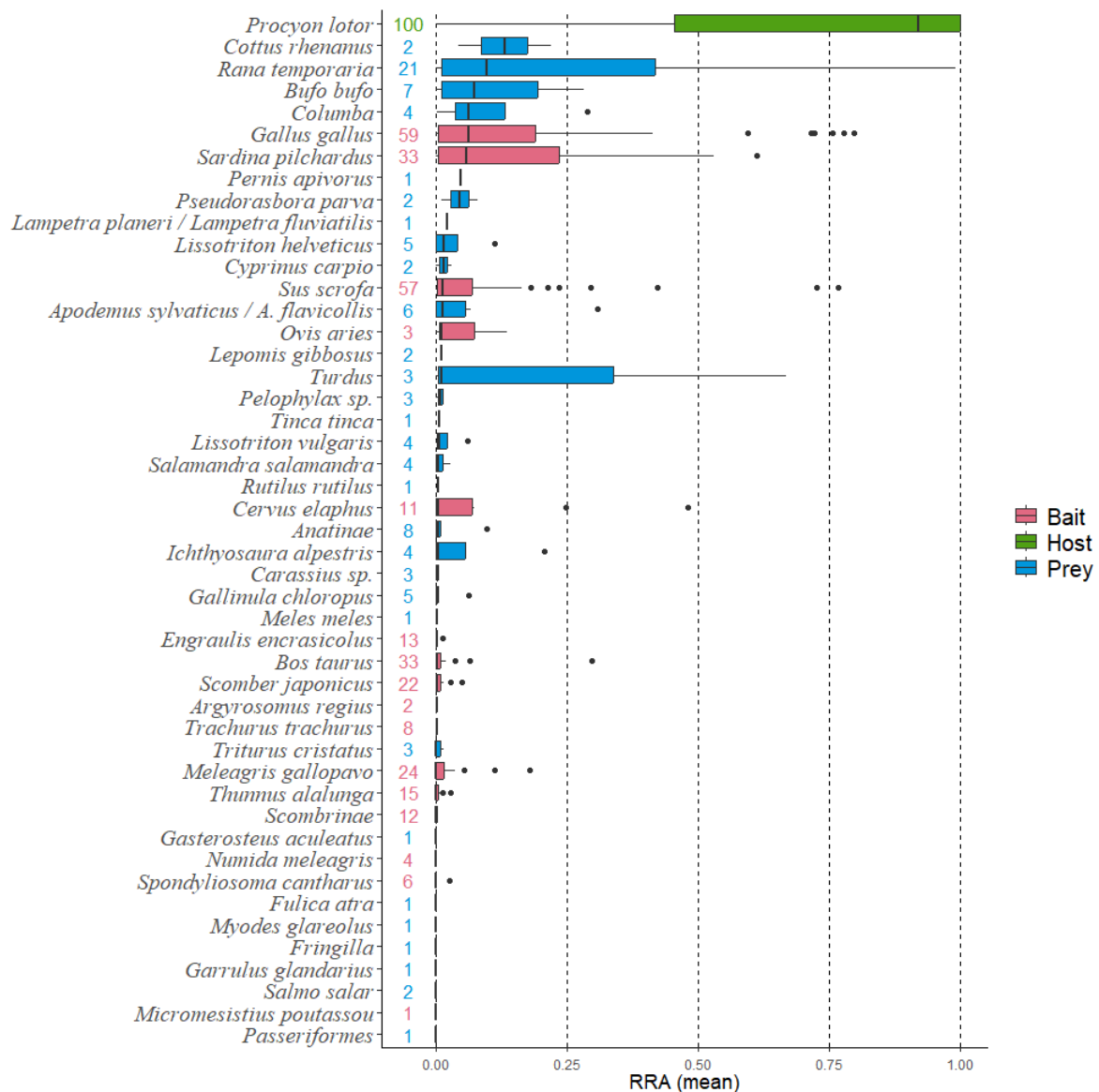


**Figure 10.** Per species boxplots of the mean Relative Read Abundance (RRA) per sample. Only samples containing the species (respective number is indicated at the base of the plot) are used in calculating summary statistics. Boxplots span from 1st to 3rd quantile, with the median value indicated as a line. Outliers are presented as scatter points. Boxplots are ordered on decreasing median value.

### 3.2 Diet composition

To focus the analysis on ecologically relevant dietary signals, raccoon host reads were excluded and the remaining non-raccoon taxa were examined to distinguish bait-derived from prey-derived contributions. Excluding the 17 samples containing exclusively raccoon reads yielded a reduced dataset of 83 samples that contained 46 non-raccoon taxa (see Supplementary Figure 1 in Appendix). Additionally, removing bait-derived taxa in a next step reduced the dataset further to 30 prey taxa, and the number of samples with a detectable prey signal to just 51 (47% of the initial 107 samples). All detected prey species and their mean RRA within all prey-positive samples are shown in Table 3.

**Table 3.** Relative Read Abundance (RRA) and prevalence of the 30 prey taxa across the 51 samples with a detectable prey signal, shown at species (or genus) level.

| Species name                                       | Prevalence | RRA (mean)     |
|----------------------------------------------------|------------|----------------|
| <i>Rana temporaria</i>                             | 21         | 0.3601516942   |
| <i>Anatinae</i>                                    | 8          | 0.07006096468  |
| <i>Bufo bufo</i>                                   | 7          | 0.08004406976  |
| <i>Apodemus sylvaticus</i> / <i>A. flavicollis</i> | 6          | 0.07474096897  |
| <i>Gallinula chloropus</i>                         | 5          | 0.05207177912  |
| <i>Lissotriton helveticus</i>                      | 5          | 0.047236054    |
| <i>Columba</i>                                     | 4          | 0.02863302544  |
| <i>Salamandra salamandra</i>                       | 4          | 0.02144303173  |
| <i>Lissotriton vulgaris</i>                        | 4          | 0.0200491531   |
| <i>Ichthyosaura alpestris</i>                      | 4          | 0.01835366884  |
| <i>Turdus</i>                                      | 3          | 0.03980007433  |
| <i>Triturus cristatus</i>                          | 3          | 0.02196412793  |
| <i>Carassius sp.</i>                               | 3          | 0.008551966437 |
| <i>Pelophylax sp.</i>                              | 3          | 0.001817925306 |
| <i>Cyprinus carpio</i>                             | 2          | 0.02364719     |
| <i>Salmo salar</i>                                 | 2          | 0.0205685551   |
| <i>Pseudorasbora parva</i>                         | 2          | 0.02038242696  |
| <i>Cottus rhenanus</i>                             | 2          | 0.005535851896 |
| <i>Lepomis gibbosus</i>                            | 2          | 0.002824623843 |
| <i>Fringilla</i>                                   | 1          | 0.01960784314  |
| <i>Meles meles</i>                                 | 1          | 0.01960784314  |
| <i>Pernis apivorus</i>                             | 1          | 0.01704364783  |
| <i>Tinca tinca</i>                                 | 1          | 0.01684004527  |

[illegible]



Several remarkable species were detected in the raccoon faecal samples. Both the fire salamander (*Salamandra salamandra*) and the great crested newt (*Triturus cristatus*) are particularly noteworthy amphibian species, due to their rarity and protected status (Figure 11a-b). Both species have multiple observations: the fire salamander was detected in four samples whereas the great crested newt was detected in three samples. The inclusion of the fire salamander in the raccoon's diet is surprising, given that this species is considered toxic. Another remarkable observation was the detection of *Lampetra planeri* / *Lampetra fluviatilis* (Figure 11c). Based on the metabarcoding primer used in this study, it is not possible to differentiate between these two fish species; however, both species are relatively rare within Belgian brook and river systems. Finally, the clear detection of European honey buzzard (*Pernis apivorus*) eDNA in considerable quantities in a single raccoon scat was surprising (Figure 11d).

The eDNA metabarcoding analysis of raccoon faeces from the Saint-Michel-Freyr massif confirmed the consumption of vertebrate fauna by at least half of the sampled individuals. Amphibians, with the common frog (*Rana temporaria*) in particular, represented the most commonly consumed vertebrate group by both abundance and prevalence. This finding is consistent with the known foraging ecology of raccoons, which are opportunistic omnivores with a well-documented preference for easily accessible prey near water bodies (Bartoszewicz et al., 2008; Libois et al., 2019; Pedlar et al., 1997). Amphibians, crustaceans, and other shoreline animals are recognised as important dietary components (Rulison *et al.*, 2012), and raccoon population densities tend to be highest in lowland forests with abundant water and marsh habitats (Fritzell, 1978). The strong amphibian signal detected here most likely reflects both the opportunistic feeding strategy of this species and the likely proximity of sampled individuals to aquatic habitats within the study area. Indeed, *R. temporaria* are true habitat generalists that are widespread in Belgium, and can easily be found near lentic water bodies.

Larger mammals, including wild boar (*Sus scrofa*), European badger (*Meles meles*) and Red deer (*Cervus elaphus*), were also detected in the samples. These detections most likely reflect opportunistic scavenging on carrion rather than active predation. Because the domestic pig and wild boar have an identical 12S amplicon sequence, they could not be differentiated. Consequently, the specific contribution of wild boar to the raccoon diet remains undetermined. Three detected taxa, *Sus scrofa*, Atlantic salmon (*Salmo salar*), and common carp (*Cyprinus carpio*), have natural populations in the study area while they are also frequently used in commercial dog food. Based on

the sequence read depth and signal strength, we decided to classify *Sus scrofa* as bait, while the two fish species were categorized as prey.

The detection of European badger (*Meles meles*) is remarkable in another way. *Meles meles* shares the NARacBlk target sequence with raccoon and is thus expected to also have a strongly inhibited signal. In addition, a heavy competition is expected with the overabundance of host DNA, leading to further suppression of the *Meles* signal. The *Meles meles* detections all originated from a single sample (W25-169) and was consistently picked up in all three technical replicates. This either indicates a clear *Meles* signal in this raccoon faecal sample, or suboptimal performance of the NARacBlk as previously discussed.

The detection of multiple native vertebrate species in the analysed raccoon faecal samples indicate that they are consuming local fauna when presented the opportunity. This suggests that, if not already, the expanding populations of raccoons in Belgium and Europe might pose a threat to indigenous biodiversity in the (near) future, as its dietary breadth and adaptability are central to its invasive success. While predator-prey interactions have been suggested as a potential ecological impact of raccoons in Europe, robust data on these interactions remain scarce (Bartoszewicz *et al.*, 2008; Libois *et al.*, 2019; Pedlar *et al.*, 1997). The present study provides a direct, molecularly verified record of native vertebrate DNA detected in raccoon faeces collected in two Walloon forests, contributing to this currently limited evidence base.

While this study presents a baseline analysis of raccoon diet via a general genetic marker, certain limitations should be kept in mind.

First, by using a vertebrate-specific marker important contributions or changes in the non-vertebrate diet remain hidden. As the raccoon is a versatile consumer, a significant proportion of their diet might consist of vegetal food sources like seeds, nuts and berries, and non-vertebrate animal species like worms, molluscs, snails, insects, crayfish, ... (Libois *et al.*, 2019).

Second, the used 12S marker is only intended to allow interpretation for mammal, fish and amphibian taxa, leaving bird detections largely uncertain. For the species that can be reliably detected with the used marker, the overabundance of host DNA further limits the detection rate and as such the number of significant inferences that can be made. Even with the incorporation of a host specific blocking nucleotide, the proportion of non-host reads in the dataset was only 25%. In addition, by using a blocking nucleotide, caution has to be made with interpreting detections of taxa with a (quasi) identical DNA sequence to that of the host, as these will be affected in the same way by the blocking agent (Ando *et al.*, 2020).

Third, DNA metabarcoding on faecal samples does not allow determination of how the host consumed its diet, nor does it allow to identify the life stage/state of the consumed species. While we present the detected diet species as the “prey” class, this does not necessarily mean that each observation was the result of an active predation. A detection could be derived from the active predation of adults or juveniles/eggs, but also from scavenging animal remains, or even from interactions with human food waste.

Fourth, a substantial number of samples only contained bait and/or host DNA (n=49). This might

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indicate that the raccoon (i) preyed upon vertebrate species, but a significant time ago so that digestion hampered the recovery of prey DNA, (ii) consumed native species, but only non-vertebrates (see above), (iii) consumed non-natural food resources (human food waste, pet food, bait, ...) or (iv) prey vertebrate DNA was present but at quantities below the detection thresholds, for instance due to a low initial proportion of prey-derived DNA relative to host and/or bait DNA. A dietary eDNA metabarcoding approach does not allow to differentiate between these scenarios.

Finally, the experimental design does not allow interpretation of separate time and location effects, limiting the study subject to a general overview of vertebrate diet composition in Wallonia during a 1 year period. In addition, some unknowns regarding the sample collection need to be taken into consideration when interpreting the data. Differences in the bait composition, the used trapping methods (cage versus paw-traps) and the faecal sample extraction/collection methods (dissection versus non-lethal extraction) are some factors that might alter the interpretation of differences between samples/sampling campaigns as much as the natural diet.

This study delivers molecularly verified characterisation of the vertebrate raccoon diet in the Walloon forests of Belgium. Using a standardised eDNA metabarcoding workflow adapted with a host-specific blocking primer, 30 vertebrate prey taxa were identified across 100 faecal samples spanning three sampling campaigns. The common frog (*Rana temporaria*) emerged as the most detected native species by both abundance and prevalence in the studied faecal samples, with birds, freshwater fish and small mammals as secondary components, suggesting that raccoons in these forests consume the most commonly available and easy to find food items. These insights should be combined with complementary research on aspects such as raccoon population densities, consumed biomass, prey availability, predation versus scavenging behavior, and prey species population trends to further characterise the effective ecological impact of vertebrate species predation by this invasive alien species in Walloon forest ecosystems. In addition, the application of invertebrate-specific markers in future work will broaden the scope of dietary characterisation for this ecologically versatile predator.

Ando H., Mukai H., Komura T., Dewi T., Ando M. & Isagi Y. (2020). Methodological trends and perspectives of animal dietary studies by noninvasive fecal DNA metabarcoding. *Environmental DNA* 2. <https://doi.org/10.1002/edn3.117>.

Bartoszewicz M., Okarma H., Zalewski A. & Szczęśna J. (2008). Ecology of the Raccoon (*Procyon lotor*) from Western Poland. *Annales Zoologici Fennici* 45: 291–298. <https://doi.org/10.5735/086.045.0409>.

Brys R., Haegeman A., Halfmaerten D., Neyrinck S., Staelens A., Auwerx J. & Ruttink T. (2020). Monitoring of spatiotemporal occupancy patterns of fish and amphibian species in a lentic aquatic system using environmental DNA. *Molecular Ecology* 30: 3097–3110. <https://doi.org/10.1111/mec.15742>.

Colleye O., Ovidio M., Salmon A. & Parmentier E. (2013). Contribution to the study of acoustic communication in two Belgian river bullheads (*Cottus rhenanus* and *C. perifretum*) with further insight into the sound-producing mechanism. *Frontiers in zoology* 10: 71. <https://doi.org/10.1186/1742-9994-10-71>.

Everts T., Van Driessche C., Sabrina N., Regge N., Descamps S., De Vocht A., Jacquemyn H. & Brys R. (2022). Using quantitative eDNA analyses to accurately estimate American bullfrog abundance and to evaluate management efficacy. *Environmental DNA* 4. <https://doi.org/10.1002/edn3.301>.

Fritzell E.K. (1978). Habitat Use by Prairie Raccoons during the Waterfowl Breeding Season. *The Journal of Wildlife Management* 42 (1): 118–127. <https://doi.org/10.2307/3800698>.

Halfmaerten D., Verschelde P., Haegeman A., Neyrinck S., Verschaeve L., Van Breusegem, A., Van Liefferinge N., Geeraerts C., Van Driessche C. & Brys R. (2023). 12S metabarcoding reference data from the Research Institute for Nature and Forest (INBO). Zenodo. <https://doi.org/https://doi.org/10.5281/zenodo.10422227>.

Haubrock P., Everts T., Abreo N.A., Bojko J., Deklerck V., Dickey J., Franco A.C., García-Berthou E., Katsanevakis S., Kirichenko N., Mammola S., Nuñez M., Parker B., Scalera R., Soto I., Strubbe D., Tarkan A., Vilizzi L., Adriaens T. & Britton R. (2025). The impacts of biological invasions. *Biological reviews of the Cambridge Philosophical Society* 101. <https://doi.org/10.1002/brv.70124>.

Krawczyk A., Bogdziewicz M., Majkowska K. & Głazaczow A. (2016). Diet composition of the Eurasian otter *Lutra lutra* in different freshwater habitats of temperate Europe: A review and meta-analysis. *Mammal Review* accepted. <https://doi.org/10.1111/mam.12054>.

Libois R., Schockert V., Lambinet C., Richet J., Campos Martinez I., Levert A. & Gautherot J. (2019). Trophic niche of three carnivores in southern Belgium : raccoon (*Procyon lotor*), European badger (*Meles meles*) and stone marten (*Martes foina*). Université de Liège.

Loupe V., Leroy B., Herrel A. & Veron G. (2019). Current and future climatic regions favourable for a globally introduced wild carnivore, the raccoon *Procyon lotor*. *Scientific Reports* 9. <https://doi.org/10.1038/s41598-019-45713-y>.

Pedlar J.H., Fahrig L. & Merriam H.G. (1997). Raccoon Habitat Use at 2 Spatial Scales. *The Journal of Wildlife Management* 61 (1): 102–112. <https://doi.org/10.2307/3802419>.

Prins T., Broothaerts W., Burns M., Demšar T., Edelman S., Papazova N., Peterseil V., Taverniers I., Silva E., Baleková S., Dimbareva D., Giacomo M., Mazzara M., Sieradzki Z., Tilocca M.G., Villa

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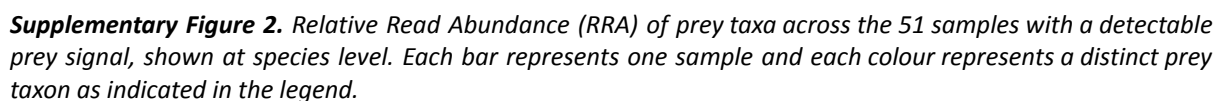


## 7 Appendices

## 7.1 Supplementary figures

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## 7.2 Supplementary tables

**Supplementary Table 1.** All detected taxa (n=47) in this study were assigned to categories based on known raccoon diet compositions and species ecology (i.e. host, bait or prey).

| Species name                                          | Class name  | Species category | Diet category |
|-------------------------------------------------------|-------------|------------------|---------------|
| <i>Apodemus sylvaticus</i> / <i>A. flavicollis</i>    | Mammalia    | Mammals small    | Prey          |
| <i>Argyrosomus regius</i>                             | Actinopteri | Fish marine      | Bait          |
| <i>Bos taurus</i>                                     | Mammalia    | Mammals big      | Bait          |
| <i>Bufo bufo</i>                                      | Amphibia    | Amphibians       | Prey          |
| <i>Carassius sp.</i>                                  | Actinopteri | Fish freshwater  | Prey          |
| <i>Cervus elaphus</i>                                 | Mammalia    | Mammals big      | Bait          |
| <i>Cottus rhenanus</i>                                | Actinopteri | Fish freshwater  | Prey          |
| <i>Cyprinus carpio</i>                                | Actinopteri | Fish freshwater  | Prey          |
| <i>Engraulis encrasicolus</i>                         | Actinopteri | Fish marine      | Bait          |
| <i>Gasterosteus aculeatus</i>                         | Actinopteri | Fish freshwater  | Prey          |
| <i>Ichthyosaura alpestris</i>                         | Amphibia    | Amphibians       | Prey          |
| <i>Lampetra planeri</i> / <i>Lampetra fluviatilis</i> | Hyperoartia | Fish freshwater  | Prey          |
| <i>Lepomis gibbosus</i>                               | Actinopteri | Fish freshwater  | Prey          |
| <i>Lissotriton helveticus</i>                         | Amphibia    | Amphibians       | Prey          |
| <i>Lissotriton vulgaris</i>                           | Amphibia    | Amphibians       | Prey          |
| <i>Meles meles</i>                                    | Mammalia    | Mammals medium   | Prey          |
| <i>Micromesistius poutassou</i>                       | Actinopteri | Fish marine      | Bait          |
| <i>Myodes glareolus</i>                               | Mammalia    | Mammals small    | Prey          |
| <i>Ovis aries</i>                                     | Mammalia    | Mammals big      | Bait          |
| <i>Pelophylax sp.</i>                                 | Amphibia    | Amphibians       | Prey          |
| <i>Procyon lotor</i>                                  | Mammalia    | Mammals medium   | Host          |
| <i>Pseudorasbora parva</i>                            | Actinopteri | Fish freshwater  | Prey          |
| <i>Rana temporaria</i>                                | Amphibia    | Amphibians       | Prey          |
| <i>Rutilus rutilus</i>                                | Actinopteri | Fish freshwater  | Prey          |
| <i>Salamandra salamandra</i>                          | Amphibia    | Amphibians       | Prey          |
| <i>Salmo salar</i>                                    | Actinopteri | Fish freshwater  | Prey          |
| <i>Sardina pilchardus</i>                             | Actinopteri | Fish marine      | Bait          |
| <i>Scomber japonicus</i>                              | Actinopteri | Fish marine      | Bait          |
| <i>Scombrinae</i>                                     | Actinopteri | Fish marine      | Bait          |

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**Figure 1.** Distribution of the northern raccoon (*Procyon lotor*) in Europe. Both records of established (reproduction) and invasive (range expansion) populations were used to build the species distribution map. The striped area represents the raccoon-invaded range in central Europe estimated by Lutz (1984). Figure is adapted from Salgado, 2018..... 6

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