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RESEARCH ARTICLE

Environmental DNA metabarcoding reveals temporal dynamics but functional stability of arthropod communities in cattle dung

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Abstract

1. Terrestrial invertebrates are highly important for the decomposition of dung from large mammals. Mammal dung has been present in many of Earth's ecosystems for millions of years, enabling the evolution of a broad diversity of dung-associated invertebrates that process various components of the dung. Today, large herbivorous mammals are increasingly introduced to ecosystems with the aim of restoring the ecological functions formerly provided by their extinct counterparts. However, we still know little about the ecosystem functions and nutrient flows in these rewilded ecosystems, including the dynamics of dung decomposition. In fact, the succession of insect communities in dung is an area of limited research attention also outside a rewilding context.
2. In this study, we use environmental DNA metabarcoding of dung from rewilded Galloway cattle in an experimental set-up to investigate invertebrate communities and functional dynamics over a time span of 53 days, starting from the time of deposition.
3. We find a strong signal of successional change in community composition, including for the species that are directly dependent on dung as a resource. While several of these species were detected consistently across the sampling period, others appeared confined to either early or late successional stages. We believe that this is indicative of evolutionary adaptation to a highly dynamic resource, with species showing niche partitioning on a temporal scale. However, our results show consistently high species diversity within the functional groups that are directly dependent on dung.
4. Our findings of such redundancy suggest functional stability of the dung-associated invertebrate community, with several species ready to fill vacant niches if other species disappear. Importantly, this might also buffer the ecosystem functions related to dung decomposition against environmental change. Interestingly, alpha diversity peaked after approximately 20–25 days in both

meadow and pasture habitats, and did not decrease substantially during the experimental period, probably due to preservation of eDNA in the dung after the disappearance of visiting invertebrates, and from detection of tissue remains and cryptic life stages.

KEYWORDS
arthropods, cattle dung, colonization, dung beetles, dung degradation, ecosystem functions, eDNA metabarcoding, succession

1 | INTRODUCTION

Many invertebrates are associated with dung from large mammal herbivores, with dung beetles and flies making up a substantial proportion of this diversity (Skidmore, 1991). Losses of large wild herbivore populations, urbanization and shifts from extensive grazing to more intensive agricultural systems have resulted in resource depletion for dung-associated invertebrates in many areas, consequently resulting in downsizing, extirpations and population declines (Carpaneto et al., 2007; Lobo, 2001; Schweiger & Svenning, 2018; Smith et al., 2018; Tonelli et al., 2018). However, (re-)introductions of large herbivores are increasingly being applied across temperate biomes to create self-regulating, biodiverse ecosystems (Pedersen et al., 2020; Svenning et al., 2016). In such systems, the functions provided by dung-associated invertebrates continue to be relevant. The presence of dung-associated beetles increases dung removal (Evans et al., 2019; Lee & Wall, 2006a; Pecenka & Lundgren, 2018; Stanbrook & King, 2022), consequently increasing nutrient cycling and ecosystem functioning. Furthermore, dung beetles reduce methane emissions from pastures, primarily through aeration of internal parts of the dung pats (Penttilä et al., 2013).

As a result of their intense competition for an ephemeral resource, dung-associated species have co-evolved to show successional and seasonal differences in activity (Floate, 2011; Sladecek et al., 2013). Several studies have investigated such successional patterns in appearance of dung-associated arthropods, but most have been restricted to certain taxonomic groups. The vast majority have investigated coprophagous beetles (Gittings & Giller, 1998; Menéndez & Gutiérrez, 1999; Rentz & Price, 2016; Wassmer, 2020), some have looked at predatory beetles (Guimarães & Mendes, 1998; Sladecek et al., 2013; Sladecek, Zitek, et al., 2021), and a few have investigated flies (Sladecek et al., 2017), or flies and beetles in combination (Lee & Wall, 2006b). In general, these studies found that flies are early colonizers, followed by coprophagous beetles, and finally predators, facilitated by the appearance of prey species belonging to the former groups. However, several factors were found to affect the order of arrival, such as emission of volatile compounds (Sladecek, Dötterl, et al., 2021), wet weight and patch size of the dung pat (Finn & Giller, 2000), and whether the dung pats were situated in open or shaded areas (Horgan, 2002). Furthermore, studies have shown priority effects with phylogenetically distant species facilitating each other, as well as exclusion effects where closely related species cannot co-exist (Sladecek, Segar, et al., 2021). These findings suggest that successional patterns are context-dependent and that a versatile, multi-taxon approach replicated over many study systems should be performed to obtain a comprehensive understanding of heterotrophic succession in mammal herbivore dung.

Recently, environmental DNA (eDNA) metabarcoding has been used to comprehensively characterize dung-associated invertebrate communities (Sigsgaard et al., 2021; Thomassen et al., 2023). This includes detection of elusive groups such as mites, collembolans and small beetles (e.g. featherwing beetles; Ptilidae), which are very difficult and resource demanding to identify morphologically. Environmental DNA metabarcoding has successfully yielded comprehensive community data, making it a valuable method for characterizing successional changes in invertebrate species composition in dung pats, especially if methodological caveats are considered and handled properly (see e.g. Alberdi et al., 2018; Beng & Corlett, 2020).

While we expect a constant turnover of species, the composition and diversity of functional groups might be expected to be more stable, as many species carry out similar functions. Functional redundancy of species has been shown to generally enhance ecological stability and resilience (Biggs et al., 2020), and high functional diversity of dung beetles specifically has been shown to increase dung removal (Milotić et al., 2019). Slade et al. (2017) showed high resilience to species loss and changing community composition for dung-beetle communities when assessed over multiple functions, suggesting that such functional redundancy does exist. This collectively suggests that while preserving species richness per se might not be key to preserving functionality, preservation of functional diversity should be.

Here, we aim to describe how the different functional groups and species of dung-associated invertebrates are distributed along the successional gradient of dung decomposition. In an experimental set-up carried out in a rewilded area, we homogenized a large quantity of fresh cattle dung, which was subsequently divided into standardized pats and sampled for eDNA under natural succession. Furthermore, as eDNA metabarcoding is increasingly being used for biomonitoring, we also evaluate species richness patterns during succession, to guide efficient sampling strategies for applied monitoring of dung-associated invertebrates.

2 | MATERIALS AND METHODS

2.1 | Site

The experiment was conducted in the Mols Laboratory area in Denmark (56°13′36″ N, 10°34′33″ E, Figure 1). The area is owned by the Natural History Museum of Aarhus and is managed by trophic rewilding (Svenning et al., 2016), where free roaming Galloway cattle and Exmoor ponies are present throughout the year, without supplementary feeding or antiparasitic treatments. The populations are regulated in relation to the carrying capacity of the area. Body conditions of the animals are continuously monitored, and animals are removed if they are in bad condition or expected to become so in the near future. The area consists of a mixture of forests and open habitats, such as pastures and meadows.

2.2 | Experimental set-up and sample collection

Sixty kg of fresh dung was collected on 12 July 2019, by following cattle in the area and collecting fresh dung in large clean buckets lined with clean plastic bags. We collected dung immediately

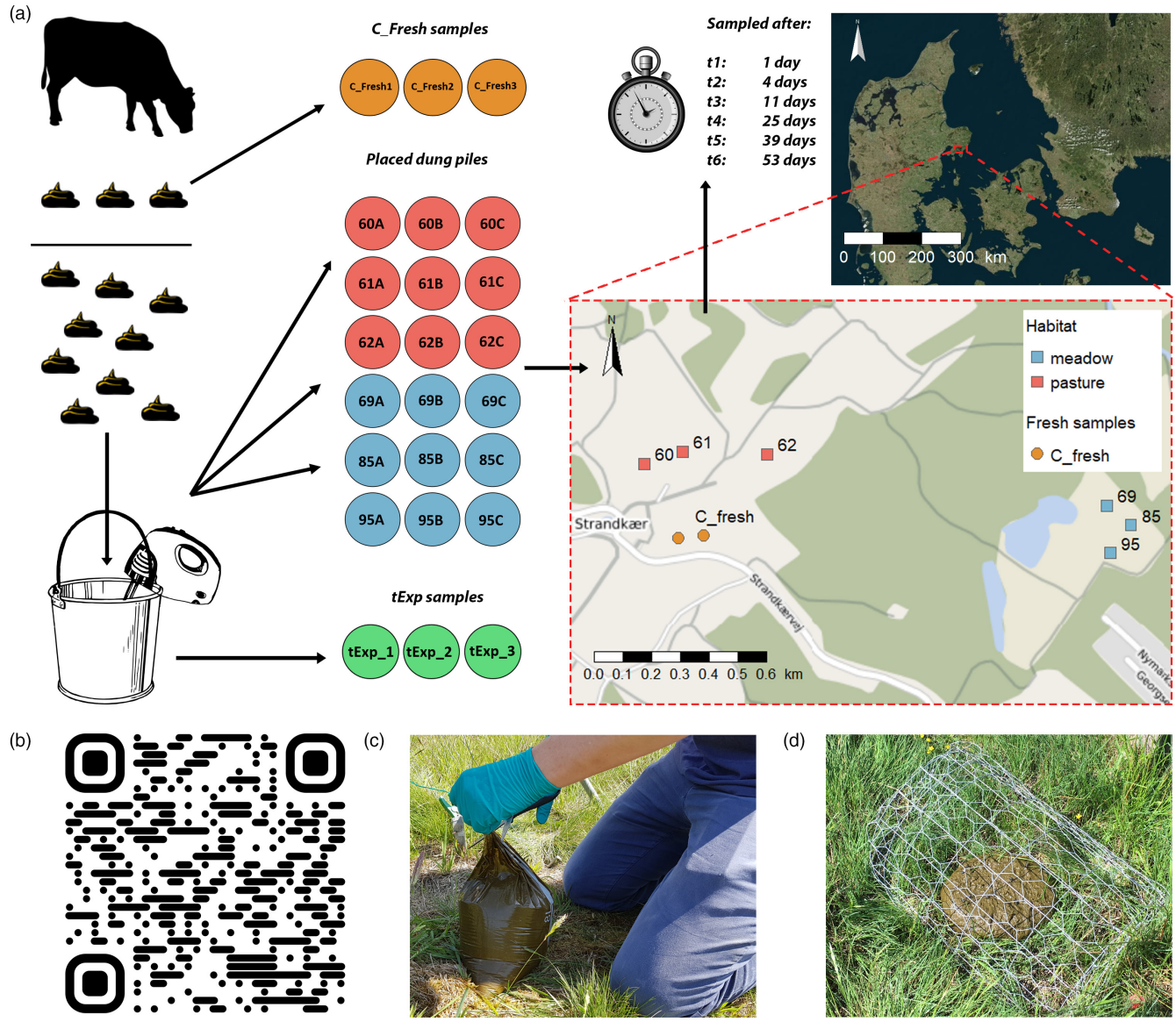

FIGURE 1 (a) Experimental design and study area. Sixty kg of fresh dung was collected by pooling dung pats together. In addition, three fresh dung pats were sampled individually (C_Fresh). Cattle were followed during dung collection, and fresh dung was collected right after deposition. The dung pool was homogenized and divided into 18 dung piles placed across six different plots (three in pastures, three in meadows). (b). QR code with link to a video showing the homogenization step. (c) The homogenized dung was divided into 18 plastic bags and subsequently placed in the six plots as shown in (a). (d) Placed dung piles, covered by nets to avoid foraging birds and mammals. Pictures and graphics: Kent Olsen; Piotr Siedlecki; openstreetmaps.org; openclipart.org; phylopic.org. QR code generated through https://www.qrcode-monkey.com/.

after visually documented deposition or from pats that looked very freshly deposited (no crust formed). The sampling was non-invasive (only dung was collected), and thus, no animal ethics approval was needed. Permission to conduct the fieldwork was given by the Natural History Museum of Aarhus. All collected dung was thoroughly homogenized by mixing it in a large plastic tub, using a mixing blade connected to a power drill, for approximately 20 min. All materials used were thoroughly cleaned with a bleach solution and 96% ethanol prior to sampling. Three samples (C_Fresh) were collected from fresh dung pats on the same day (these were not included in the homogenized pool) and included in the sequencing to elucidate the DNA community in the dung right after deposition. The 60 kg of homogenized pool of dung was separated into 18 plastic bags (homogenized dung piles), each containing 3 kg of dung. The 18 homogenized dung piles were divided between six experimental plots, with three dung piles in each plot (Figure 1). Three plots (60, 61 and 62; Figure 1) were situated in pastures, and three plots (69, 85 and 95; Figure 1) were situated in meadow habitats. Initially, three samples (tExp) were collected from the large pool of homogenized dung before it was separated into the 18 pools, to represent the starting community in the homogenized dung pool. Each of the 18 dung piles were subsequently sampled in 5-mL Eppendorf tubes by the same approach as in Sigsgaard et al. (2021; one sample: ~5 mL in total from five different locations in the pat). This was done after 1 day (t1), 4 days (t2), 11 days (t3), 25 days (t4), 39 days (t5) and 53 days (t6), respectively, resulting in 108 samples in total (Table S1, Figure 1). After the sampling at 53 days, the experiment was ended, as the cow pats were highly degraded, and very dry, and no activity was observed anymore. All samples were collected using nitrile gloves and face masks to avoid contamination, and samples were stored in a cooling bag with ice packs immediately after collection, and frozen at −20°C on the same day, after returning from the field.

2.3 | DNA laboratories and extraction

DNA extraction and PCR set-up was conducted in the clean laboratory facilities at the Department of Biology, Aarhus University. Anti-contamination protocols are in place, such as daily treatment with UV light, cleaning with DNA degrading chemicals (DNA-away) during work, weekly thorough cleaning with bleach and work is only performed wearing suits, masks, hairnets, sleeve covers and two pairs of gloves. Furthermore, PCR set-up and DNA work are separated, and PCR reactions and post-PCR work are carried out in a separate building.

The dung samples were transferred to 15-mL falcon tubes to facilitate mixing, and nuclease-free water was added to the samples, which were otherwise too dry to mix properly. In addition to making the homogenization treatment itself more standardized across samples, this practice made the samples more comparable in terms of water content. The tubes were shaken for 20 min on a vortexer at maximum speed to homogenize the samples. Approximately 220 mg of sample was transferred to a 2-mL Eppendorf tube by using sterile metal spatulas, which were cleaned in a chlorine bath prior to and between each sample treatment. DNA was extracted from the dung samples using the QIAmp Fast DNA Stool Mini Kit (Qiagen, Cat. No. 51604). The manufacturer's protocol was followed, except for the inclusion of a 2-h incubation step at room temperature after InhibitEX buffer was added, followed by 5 min of centrifugation at 14,000 rpm (18,407 *g*), and final elution in 2 × 60 µL ATE buffer, with 5 min for incubation and 1 min of centrifugation. An extraction blank was included in each round of DNA extractions, resulting in a total of 13 extraction blanks (Table S1), which were included in the sequencing libraries. The final eluates were stored at −21°C until PCR amplification.

2.4 | PCR amplification and next-generation sequencing

Invertebrate DNA was amplified using PCR with the BF-1/BR-1 primers (Elbrecht & Leese, 2017), targeting a 217 bp fragment of the COI-region of mtDNA. COI was chosen as it outperforms other universal barcodes (e.g. 18S) for species-level identifications and has performed well for characterizing dung-associated arthropods previously (Alberdi et al., 2018; Thomassen et al., 2023; Thomsen & Sigsgaard, 2019). Each primer was uniquely tagged with six-nucleotide tags designed using the OligoTag program (Coissac, 2012). To avoid tag jumps, identical tags were used for the forward and reverse primer (Schnell et al., 2015), and each tag was preceded by two–three random bases to increase sequence complexity (De Barba et al., 2014). All 114 samples (including C_Fresh and tEXP) and 13 extraction blanks were divided into three unique PCR set-ups. Nine samples from another study were included in the sequencing libraries. Each PCR set-up also included three PCR blanks, and no tags were re-used within a set-up. Each set-up was run four times, resulting in four PCR replicates of each sample (see Figure S8 for ASV accumulation curves). Annealing temperature and reaction volumes followed Sigsgaard et al. (2021) with only minor adjustments (Table S2). PCR products of each replicate were pooled, whereafter a 100 µL subsample was purified using the MinElute PCR purification kit from Qiagen. The protocol supplied by the manufacturer was followed, except in the final elution step, which was done by adding 20 µL elution buffer (EB), incubating at 37°C for 10 min, and finally centrifuging for 1 min at 7000 *g*. Library building and 150 bp paired-end (PE) sequencing on an Illumina NovaSeq 6000 platform was performed by Novogene (Cambridge, UK), requesting 10 Gb of output for each library.

2.5 | Bioinformatics and data filtering

The raw sequencing data files were run through the MetaBarFlow pipeline (Sigsgaard et al., 2022), which performed demultiplexing based on Cutadapt (Martin, 2011), quality trimming using

sickle (Joshi & Fass, 2011) and error filtering using DADA2 (Callahan et al., 2016). The parameters used followed Thomassen et al. (2023). After these filtering steps, and merging of paired reads, the resulting amplicon sequence variants (ASVs) were matched to a custom COI database, containing all eukaryotic COI sequences from the Genbank nt database (Sayers et al., 2020) and BOLD (Ratnasingham & Hebert, 2007). The database was built using the MARES pipeline (Arranz et al., 2020), and sequences downloaded 16 November 2020, with the same search terms as in Klepke et al. (2022). Database coverage was evaluated for all arthropod species observed at the study site (source: www.naturbasen.dk) by searching for species and genus names in the COI database and summarized by order and family for the two most important dung-associated orders: Coleoptera and Diptera. The BLASTn algorithm was used for matching ASVs against the database, requesting a maximum of 500 hits per ASV, minimum 90% query coverage per high-scoring segment pair and minimum 80% sequence similarity. Taxonomic identifications of all ASVs with a database match were then obtained using the R package taxizedb version 0.3.0 (Chamberlain et al., 2021). The most recent common ancestor of the taxon/taxa yielding the highest sequence similarity (e.g. 99%) and any other taxa showing sequence similarities within the range of the hits to the best-matching taxon/taxa (e.g. 98%–99%) was assigned as the final identification (Sigsgaard et al., 2021). Only hits within a 2% margin of the best hits were considered. ASVs that could not be identified to species level were assigned as 'putative species' and named using the lowest assigned taxonomic classification (e.g. Diptera), followed by 'sp.' and a running enumeration for different groups of species matches resulting in the same higher level identification (e.g. Diptera sp.1 and Diptera sp.2). Only BLAST hits with ≥90% sequence similarity and ≥97% query coverage were included in the taxonomic assignment, and species-level identification was only made if the best hit showed ≥98% sequence similarity. Identifications of all ASVs were checked for errors resulting from spurious reference database sequences (e.g. sequences with substantially better hits to other species than the one they had been assigned to), and when this was the case, the taxonomy was manually assigned by considering hits as described above but disregarding the spurious sequence. The ASVs identified to the same taxon (known or putative species) were hereafter merged.

Any taxon that was present at a higher read count in a control sample (PCR blanks and extraction blanks) than in any dung sample was removed from the data set (Table S3). If a taxon was present in only a single PCR replicate, it was removed as well. Rarefaction analyses were conducted using the rarecurve function from the R package *vegan* version 2.5-7 (Oksanen et al., 2020). PCR replicates were rarefied using the median read depth of all replicates (83,822 reads). The median was chosen as it has been shown to keep most resolution, while controlling for differences in sequencing efficiency between replicates, compared with other normalization thresholds (deCárcer et al., 2011). Subsequently, PCR replicates were aggregated, and all samples were rarefied using the minimum read depth of all aggregated samples (251,466 reads). The R package *ROBITools* version 0.1 (LECA, 2012) was used for rarefaction. Samples were summed within each plot at each time category to control for non-independence between samples from the same plot. These summed samples will hereafter be referred to simply as 'samples'. Finally, each of the identified taxa were denoted as dung-associated or not dung-associated based on the literature (Barclay & Bouchard, 2023; Lee et al., 2002; Marshall, 2012; Pechenik, 2010; Skidmore, 1991; Walter & Proctor, 2013). Furthermore, they were assigned to a functional group according to Skidmore (1991), with the inclusion of additional groups defined for this study (see Table 1 for description of groups, and Table S5 for group assignments of each species). The additional groups served to include additional organisms potentially relevant to dung decomposition, as well as non-dung-associated invertebrate taxa that may leave eDNA traces in dung, such as cow parasites. The additional groups were defined based on feeding ecology and taxonomy, using Walter and Proctor (2013) for mites, Marshall (2012) for flies and allies, Lee et al. (2002) for nematodes, and Barclay and Bouchard (2023) for beetles.

2.6 | Statistical analyses

The data set was divided into 'dung-associated' and 'not dung-associated' taxa, and species richness values were calculated separately for these two groups, including only species-level identifications. Species richness for each sample was then plotted against time since dung pile placement. For the dung-associated species, the resulting correlation was nonlinear, and thus, generalized additive mixed models (GAMM; Wood, 2017) were fitted to the data. The response was expected to follow a Poisson distribution (log-link). Habitat was included as a fixed effect, and a smoother function was defined for time in each habitat. The smoother function used penalized cubic regression splines, with penalties modified to approach zero as smoothing parameters increase (argument bs="cs"). Four basis dimensions ($k=4$) were used for the dung-associated species, and five ($k=5$) for the non-dung-associated, as this produced the highest *r*-squared values. Finally, a random effect of each plot was included to account for non-independence between subsequent samples from the same dung piles in each plot. All concurvity measures were well below levels where false-positive effects are likely (He, 2004). The R package *mgcv* version 1.8.39 (Wood, 2011) was used for fitting GAMMs. Additionally, separate GAMMs were fitted for each functional group, with the same parameters as described in the section above, and four basis dimensions included.

To evaluate changes in community composition over time and between habitats, a non-metric multidimensional scaling ordination (nMDS) was performed. The metaMDS function from *vegan* was used, with Jaccard indices (dissimilarity-measure) based on the presence–absence data as input, and two nMDS axes were included. Differences between groups were evaluated by permutational

TABLE 1 Overview of functional groups used in this study. The association with herbivore dung is indicated. Groups are based on Skidmore (1991), with the addition of 32 groups defined for the current study based on feeding ecology and taxonomy.

Functional group	Description	Dung-associated
Annelida I	Terrestrial, detrivorous annelids	Yes
Annelida II	Freshwater and marine annelids	No
Araneae I	All spiders	No
Branchiopoda I	All branchiopods	No
Coleoptera A	Beetles, whose larvae feed upon dung	Yes
Coleoptera B	Predatory beetles, hunting in dung	Yes
Coleoptera C	Fungivorous beetles	Yes
Coleoptera O	Beetles not associated with dung or plants	No
Coleoptera P	Plant-associated beetles	No
Collembola	All collembolans	Yes
Diptera D	Flies and allies; larvae copro- or saprophagous	Yes
Diptera E	Muscidae, whose larvae become carnivorous in the final instar	Yes
Diptera F	Muscidae, whose larvae are obligate carnivores	Yes
Diptera G	Flies and allies; larvae internal parasites of the animal producing dung	Yes
Diptera L	Parasitoid flies	Yes
Diptera M	Flies and allies; adults attracted to dung, but larvae develop elsewhere	Yes
Diptera O	Flies and allies not associated with dung or plants	No
Diptera P	Plant associated flies and allies	No
Diptera NA	Flies and allies; only identified at order level and thus no available function	No
Diplopoda I	All millipedes	Yes
Hemiptera I	All hemipterans	No
Hymenoptera H	Hymenopterans; larvae parasitic/parasitoids in larvae/pupae of other insects	Yes
Hymenoptera Q	Hymenopterans; not dung-associated, or associated with dung-visiting species	No
Insecta NA	Insects; only identified at class level, and thus no available function	No
Isopoda I	Woodlouse; decomposers	Yes
Lepidoptera I	All lepidoptera	No
Mites I	Predatory/parasitic/phoretic mites	Yes
Mites II	Orbatid mites	Yes
Mites III	Gall mites/other plant-associated mites	No
Mollusca I	Freshwater molluscs	No
Mollusca II	Terrestrial slugs and snails	No
Nematoda I	Nematodes; Internal parasites of mammals	Host-related
Nematoda II	Nematodes; Free-living, in the soil, decomposers	Yes
Nematoda III	Nematodes; Internal parasites of non-mammal tetrapods and arthropods	Yes
Nematoda IV	Nematodes; Free-living and saprophagous, facultative parasites	Yes
Nematoda V	Nematodes; Plant pathogens	No
Nematoda NA	Nematodes; only identified at class level, and thus no available function	No
Orthoptera I	Herbivorous grasshoppers	No
Phthiraptera I	External parasites of mammals, Phthiraptera	Host-related
Rotifera I	All rotiferans	No
Tardigrada I	All tardigrades	Yes
Thysanoptera I	All thrips	No

analysis of variance (PERMANOVA, number of permutations = 999), using the adonis2 function from *vegan* on the Jaccard indices. Time (t1–t6) and habitat (meadow/pasture) were used as predictors.

For each habitat, ANOVA with subsequent t-tests were used to test for differences in Jaccard indices between pairs of adjacent time points (e.g. t1 and t2 and t2 and t3). Dissimilarities between all pairs

of adjacent timepoints were compared within habitats, and between habitats within each pair of time points (e.g. dissimilarities between t1 and t2 for meadow samples were compared with dissimilarities between t1 and t2 for pasture samples). All *p*-values were adjusted using the Holm–Bonferroni method, to minimize the risk of false positives due to multiple comparisons (Holm, 1979).

Finally, a multivariate binomial regression analysis was performed to estimate the probability of sampling different species as a function of habitat and time. For this analysis, time was divided into two categories (early: t1, t2 and t3; late: t4, t5 and t6) to compare the early successional stages with the later ones. The simper function from *vegan* was used to identify which species contributed most to the difference in species composition between early and late samples. The species which jointly accounted for 80% of the accumulated variance in time (Figure S5) were selected and included in the model. Species appearing in fewer than six samples were removed to avoid false-positive effects due to low detection rates. The detections of these species are shown in Figure S4. A generalized linear latent variable model was fitted using the *gllvm* package (Niku et al., 2019), specifying the presence–absence matrix as the response (assuming a binomial distribution), and with time (early/late) and habitat (meadow/pasture) as predictors. A random row effect for each plot was included, to handle non-independence between subsequent samples from the same plot. The optimal number of latent variables was evaluated by AIC values, and the best model fit was obtained by including one latent variable.

3 | RESULTS

3.1 | Sequencing output and database coverage

In total, 854,622,862 raw reads were obtained from the NovaSeq platform, with a sequencing depth per library of 71,218,572 ± 2,748,562 (mean ± SD). After merging of paired reads, cleaning and filtering, and subtraction of the samples from the other study included in the sequencing libraries, 154,089,442 reads remained. After subtracting ASVs identified as hartebeest (*Alcelaphus*), cattle (*Bos*) and non-metazoans, a taxonomic identification could be made for 11,641 ASVs, corresponding to 58,999,268 reads. After filtering based on negative controls, removal of sequences appearing in only one PCR replicate, rarefaction and aggregation of PCR replicates, 28,667,124 reads remained. These final reads represented 122 families, 218 genera and 276 species (Table S5), of which 56 families (46%), 121 genera (56%) and 172 species (62%) are known to colonize dung. Species-level database coverage for arthropod orders with dung-associated species were >75% for most orders, with only Diptera and Prostigmata having high number of unrepresented species (33% and 77% missing, respectively, Figure S11A). Genus-level coverage was >96% for all orders (Figure S11B). Within Coleoptera, coverage at species-level was >89% for all families with dung-associated species, except for Histeridae, where 34% of species were missing (Figure S11C). Within Diptera, several families had species-level coverages <60% (Figure S11E), but genus-level coverage was >94% for all families of Coleoptera and Diptera with dung-associated species (Figure S11D,F).

3.2 | Presence of species in the fresh dung samples and the homogenized pool

As expected, the samples collected immediately after deposition (C_Fresh) mainly included DNA from plant-associated species and genera, and internal or external cattle parasites. Examples of plant-associated beetles and flies detected were *Altica* sp.1, *Hydrothassa glabra*, *Hypera plantaginis*, and *Drosophila* sp.1 (*ogumai*). Furthermore, DNA was detected from *Bunostomum phlebotomum*, *Cooperia oncophora*, *Necator* sp.1, *Ostertagia ostertagi* and *Trichostrongylus axei*, which represented internal parasitic nematodes, and from the external cattle parasite *Bovicola bovis* (Table S4).

Many of those species were also detected in the samples collected from the initial homogenized dung pool (tExp), along with species known to be attracted to fresh dung such as *Aphodius haemorrhoidalis*, *Hylemya vagans*, *Mesembrina meridiana*, *Myospila meditabunda*, *Neomyia cornicina*, *Scathophaga stercoraria and Sepsis cynipsea* (Table S4; Lee & Wall, 2006b; Sladecek et al., 2017).

3.3 | Successional change in species richness

Very different patterns of successional changes in species richness were found for dung colonizers compared with non-colonizing species. For dung-colonizing species, the generalized additive model showed increasing species richness from the beginning of the experiment and until ~20–25 days for both habitat types, whereafter the richness curves reached a saturation point (Figure 2a, Table 2). For non-dung-associated species, the richness decreased linearly with time (Figure 2b, Table 2). Changes in species richness were different between functional groups. Some increased fast, with subsequent saturation or a slow decrease (e.g. beetles and flies with coprophagous larvae [Coleoptera A, Diptera D]). Other groups showed a later (e.g. predatory and fungivorous beetles [Coleoptera B and C]) or slower (e.g. Collembolans, orbatid mites [Mites II]) increase, but still showed a clear tendency towards saturation. Finally, some decreased through time (e.g. mammal parasites [Nematoda I, Phthiraptera I]; Figure 3). See Figure S1 for the additional groups not included in Figure 3. The patterns of change in species richness were highly congruent between the two habitats, with only one or a few exceptions in low-abundant groups (e.g. Muscidae spp. whose larvae become carnivorous in the final instar [Diptera E]).

3.4 | Successional change in species compositions and habitat differences

The nMDS analysis showed a reasonably good fit (stress = 0.1071), and homogenous dispersion between groups (permutation tests,

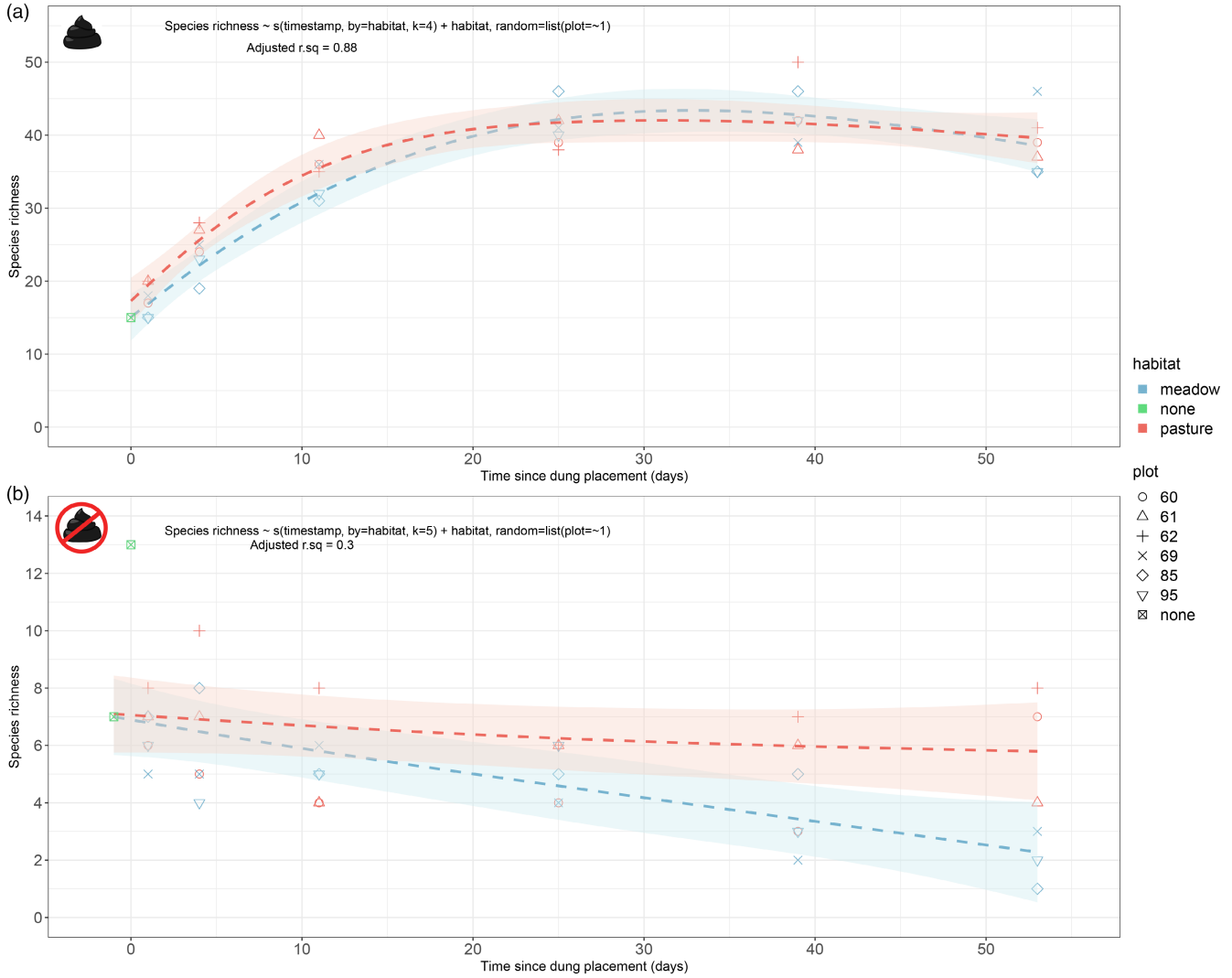

FIGURE 2 Species richness as a function of time since dung placement of dung-colonizers (a) and non-dung-colonizers (b). Generalized additive mixed models (GAMMs) have been fitted to describe species richness over time since dung placement in the two different habitat groups. Transparent areas represent the 95% confidence intervals for the model estimates. Graphical insets with minor edits from openclipart.org.

$p = 0.15$). Samples from the two habitats and samples from different time points were clearly separated, with a tendency of decreasing dissimilarity with time (Figure 4A,B). The ordination locations for species within some of the dung-associated groups is shown in Figure S2. Community dissimilarities were normally distributed (Shapiro–Wilks Test, p-value = 0.4), and generally larger between adjacent sampling points in the early successional timepoints (t1, t2 and t3) compared with the later time points (t4, t5 and t6) (t-tests, adjusted $p < 0.05$, Figure 4C, Table S7). The only exception was for t2 and t3 from the pasture habitat, which were not significantly different from any other groups (t-tests, adjusted $p > 0.1$, Figure 4C, Table S7).

PERMANOVA tests showed that 41.7% of variation between samples could be explained by time since deposition, whereas 6.8% was explained by habitat (Table 2). The majority of species (69 species) were present in both habitats, but six species were only found in meadow samples, and 11 species were only found in pasture samples (Figure 4D). A few species were found in both habitat types but still showed an association with habitat. In pasture habitat, the flies *Chloromyia formosa*, *N. cornicina* and *Crossopalpus humilis*, the mite *Uropoda orbicularis* and the nematode *Micoletzkya* sp.2 were found with higher probability (Figure S6). In meadow habitat, this was the case for the rove beetle *Philonthus albipes*, the featherwing beetle *Ptenidium nitidum*, the collembolan *Desoria grisea* and the fly *Bradysia pallipes* (Figure S6, Table S6).

3.5 | Succession of functional groups and species relevant for dung decomposition

Some functional groups were represented throughout the sampling period and showed no obvious changes over time (e.g. Coleoptera A, Diptera D and Collembola), while others were clearly associated with the late successional stages (Coleoptera B

TABLE 2 Model outputs.

Model	Input	Formula				
GAMM	Dung-colonizers	SR ~ s(time, by = habitat, k = 4, bs="cs") + habitat				
		Parametric coefficients	**Estimate**	**Std.Error**	***t* value**	***p* value**
		Intercept	3.391	0.045	76.09	**<2e-16*** **
		Habitat – pasture	0.067	0.062	1.09	0.29
		Smooth terms	**Edf**	**Rel.df**	***F* value**	***p* value**
		s(time):meadow	2.42	3	20.9	**<2e-16*** **
		s(time):pasture	2.46	3	15.2	**2.8e-6*** **
		Random effects	**SD**			
		(Intercept|plot)	3.59e-6			
GAMM	Non-dung-colonizers	SR ~ s(time, by = habitat, k = 5, bs="cs") + habitat				
		Parametric coefficients	**Estimate**	**Std.Error**	***t* value**	***p* value**
		Intercept	1.574	0.133	11.83	**<2e-16*** **
		Habitat – pasture	0.303	0.175	1.73	0.093
		Smooth terms	**Edf**	**Rel.df**	***F* value**	***p* value**
		s(time):meadow	1.204	4	2.38	**0.004****
		s(time):pasture	0.102	4	0.03	0.327
		Random effects	**SD**			
		(Intercept | plot)	0.1491			
PERMANOVA	Dung-colonizers	Jaccard indices ~ timepoint * habitat (999 permutations)				
		Parameter	**df**	**R^2**	***F* value**	***p* value**
		Timepoint	5	0.417	4.757	**0.001*** **
		Habitat	1	0.068	3.873	**0.001*** **
		Time: habitat	5	0.094	1.068	0.318
		Residual	24	0.421		
		Total	35	1		
GLLVM	Species chosen by simper	Presence-absence of each species ~ habitat+time(early/late), row.eff = ~(1|plot)				
		Coefficients	**Estimate**	**Std.Error**	***z* value**	***p* value**
		Species: time	See Figure 6 and Table S6			
		Species: habitat	See Figure S9 and Table S6			

Abbreviation: SR, species richness.

Bold values and asterisks denote significant *p* values using a significance-level of 0.05. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

and C; Figure 5 and Figure S10). Only Diptera E seemed to occur most often in the early stages of decomposition, but this pattern should be interpreted with caution due to Diptera E being a low-abundant group (Figure 5).

As for individual species, some were found to have a higher probability of presence in early successional stages than the late ones (Figures 5 and 6, Table S6). Examples include the collembolan *Sminthurus viridis*, the flies *H. vagans*, *S. stercoraria* and *N. cornicina*, the nematode *T. axei* and the louse *B. bovis*. More species were found with higher probability in late successional stages than in early (Figures 5 and 6, Table S6). These included water scavenger beetles (*Cryptopleurum minutum*, *Cercyon pygmaeus*), rove beetles (*P. albipes*, *Atheta sordidula*), featherwing beetles (*Acrotrichis sericans*, *Ptilium exaratum*), collembolans (*Parisotoma notabilis*, *D. grisea*), a soldier fly (*C. formosa*), a fungus gnat (*Bradysia flavipila*), a non-biting midge (*Camptocladius stercorarius*), a moth fly (*Psychoda setigera*), a parasitic mite (*U. orbicularis*), nematodes that are parasites of mammals (*Quilonia* sp.1, *Perostrongylus* sp.1) and two unidentified species of arthropod parasites from the nematode genus *Acrostichus*.

Finally, several species were detected at every time point (Figure 5). These included coprophagous beetles belonging to the group Coleoptera A (*A. haemorrhoidalis*, *Sphaeridium* sp.2 [*lunatum/bipustulatum*]), coprophagous flies from Diptera D (*Sepsis duplicata*, *Sepsis* sp.4 [*flavimana/neocynipsea*], *S. cynipsea*, *Psychoda trinodulosa*, *Psycoda phalaenoides*, *Musca autumnalis*), the collembolan *Ceratophysella denticulata*, a parasitic mite from the group Mites I (*Macrocheles glaber*), and a saprophagous nematode from Nematoda IV (*Pelodera* sp.1). For some of these species, their relative read proportions through time suggested associations with early successional stages as well (Figure S9). This included *Sphaeridium* sp.2 (*lunatum/bipustulatum*), *C. denticulata*, *Sepsis* sp.4

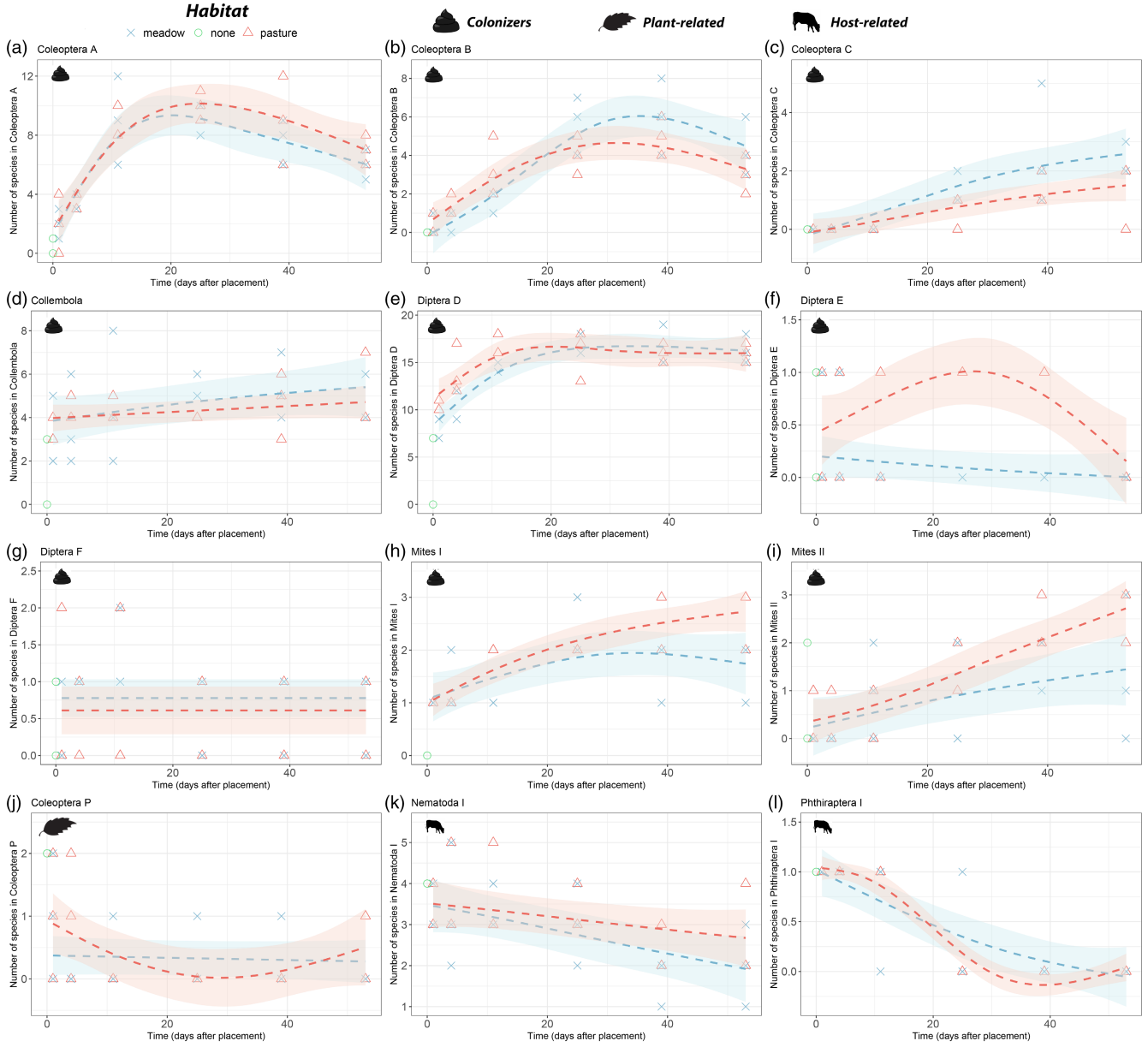

FIGURE 3 Species richness as a function of time since dung placement of a selection of the functional groups included in this study (Table 1). a) Coleoptera A, b) Coleoptera B, c) Coleoptera C, d) Collembola, e) Diptera D, f) Diptera E, g) Diptera F, h) Mites I, i) Mites II, j) Coleoptera P, k) Nematoda I, l) Phthiraptera I. The rest of the functional groups are shown in Figure S1. A generalized additive mixed model (GAMM) is fitted for each habitat (red = pasture; blue = meadow) for each functional group. The symbol in the upper left corner denotes the association with dung for species in the respective functional group (see legend for description). Graphics from openclipart.org & phylopic.org.

(*flavimana/neocynipsea*), *M. autumnalis*, *P. trinodulosa* and *P. phalaenoides*. For *S. cynipsea* and *S. duplicata*, patterns of relative read abundances indicated colonization at mid-successional stages (Figure S9).

4 | DISCUSSION

The relevance of grazing for shaping plant communities is well-established, but we still lack a thorough understanding of the interactions between herbivores and the co-occurring biota, especially in systems where herbivore species are (re-)introduced (Pringle et al., 2023; Svenning et al., 2016; Vera, 2000). Arthropod succession in mammal herbivore dung has mainly been investigated for beetles and flies but not yet with a multi-taxon approach (Lee & Wall, 2006b; Menéndez & Gutiérrez, 1999; Sladecek et al., 2017). Here, we use eDNA metabarcoding to describe successional patterns in dung for a broad array of invertebrate taxa, including elusive species, such as collembolans, mites, nematodes and small fungivorous beetles. Our findings demonstrate continuous turnover in

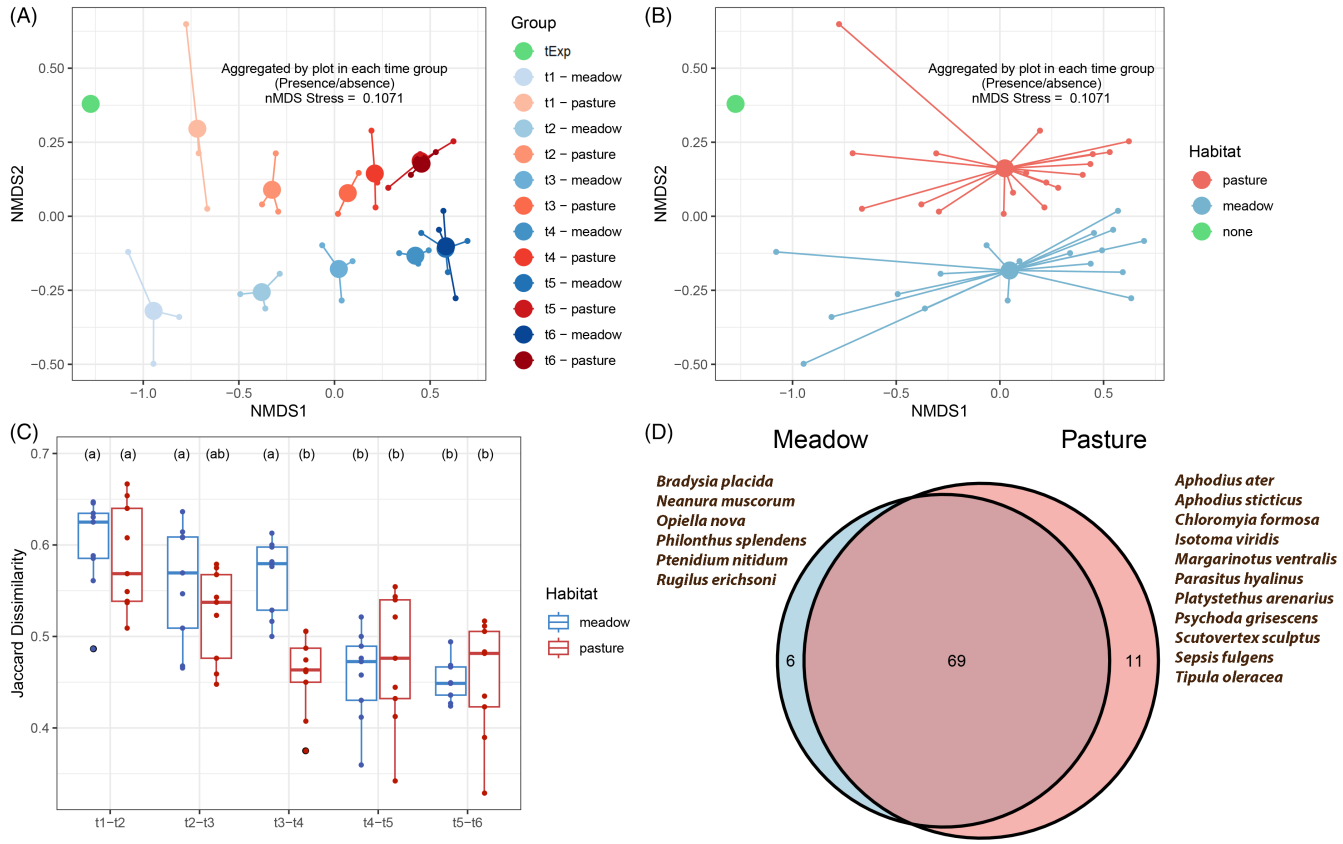

FIGURE 4 (A and B) Non-metric multidimensional scaling (NMDS) plots of all samples. (A) Separated by time point in each habitat, (B): separated by habitat. NMDS analysis was based on Jaccard indices using presence–absence data. Two NMDS dimensions were included. (C) Dissimilarity plot showing dissimilarities between groups of adjacent timepoints. Letters (a, b, ab) denote groupings based on pairwise comparisons (*t*-tests, with Holm–Bonferroni corrected *p*-values), that is, sample groups with the same letter were not significantly different (alpha = 0.05). (D) Venn diagram showing the species uniquely found in one habitat type, and the species shared between habitats, after all species with uncertain identification (e.g. Diptera sp.206, Anopheles sp.4) and all species occurring in less than three samples had been removed.

community compositions, with the highest rate of change in the initial phase of dung decomposition. Furthermore, we found that functional groups directly related to dung decomposition, such as dung beetles and flies, were present throughout the study period, while functional groups of higher trophic levels, such as predatory and fungivorous beetles, were confined to later temporal stages. Importantly, we found many cases of species being replaced by other, functionally similar species over the period of the study. We speculate that such functional redundancy might serve as a buffering mechanism for decomposition in a changing environment. Our findings represent the first successional investigations of dung using a DNA-based multi-taxon approach and are highly relevant for applied biomonitoring of dung-associated fauna and for improving our understanding of terrestrial ecosystem functioning.

4.1 | Successional order of species and functional groups

For functional groups, our data confirmed previous findings with initial colonization by flies (Diptera D, E & F) and coprophagous beetles (Coleoptera A), followed by predatory beetles (Coleoptera B; Figures 3 and 5a). However, as our first sampling was after 1 day, we might have failed to separate the initial dynamics of colonization happening in the first few hours after dung deposition (Sladecek et al., 2017). For fungivorous beetles (e.g., Coleoptera C), we found no appearances until Day 25 (t4, Figures 3 and 5a). This is expected as the arrival of this functional group likely depends on a certain minimum level of fungal growth. Parasitic mites (Mites I) and nematodes that are parasites on arthropods (Nematoda III) were detected from Day 1 (t1) and throughout the succession, probably due to their association with a range of host species arriving at different successional stages. This was also the case for collembolans (Collembola) and soil nematodes (Nematoda II and IV), suggesting that dung colonization by species from the soil happens relatively fast and continuously throughout the succession. This pattern of continuous colonization was further supported by certain collembolans (e.g. *Parisotoma notabilis* & *D. grisea*) being specifically associated with late successional stages (Figure 6). Plant-associated groups (Coleoptera P, Diptera P and Hemiptera I) were mainly present in early successional stages, and in the samples taken directly after deposition (C_Fresh), suggesting that these groups had predominantly entered the

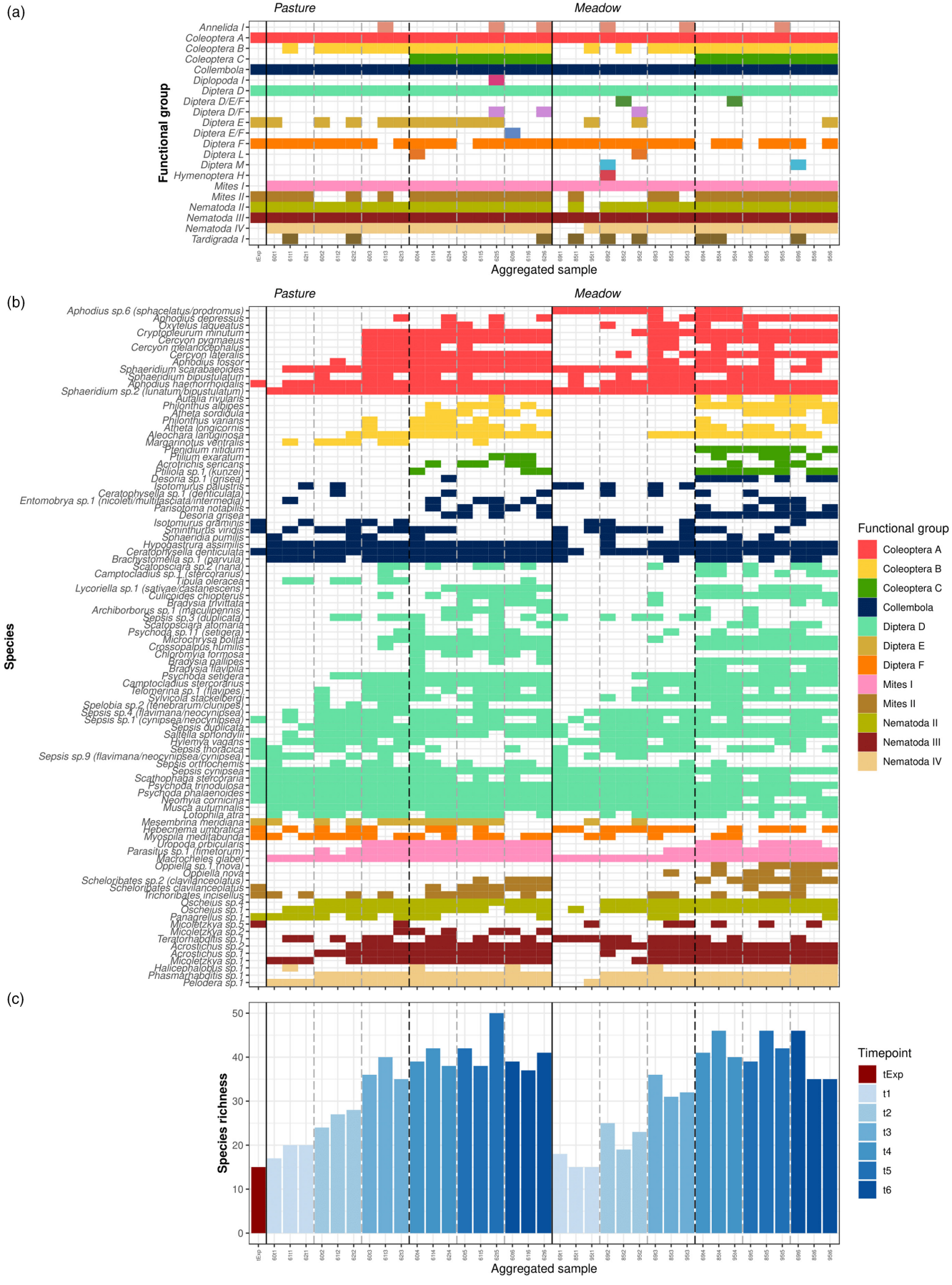

FIGURE 5 (a and b) Heatmaps showing occurrences of functional groups (a) and dung-colonizing species (b) in each sample from pasture plots (left panel) and meadow plots (right panel). Time since dung placement increases from left to right within each habitat type. Species occurrences are coloured by functional group. Only species occurring in ≥6 samples are shown. The remaining rare species are shown in Figure S4. Occurrences in the tExp samples are shown in the panel furthest to the left. (c) Species richness in each sample.

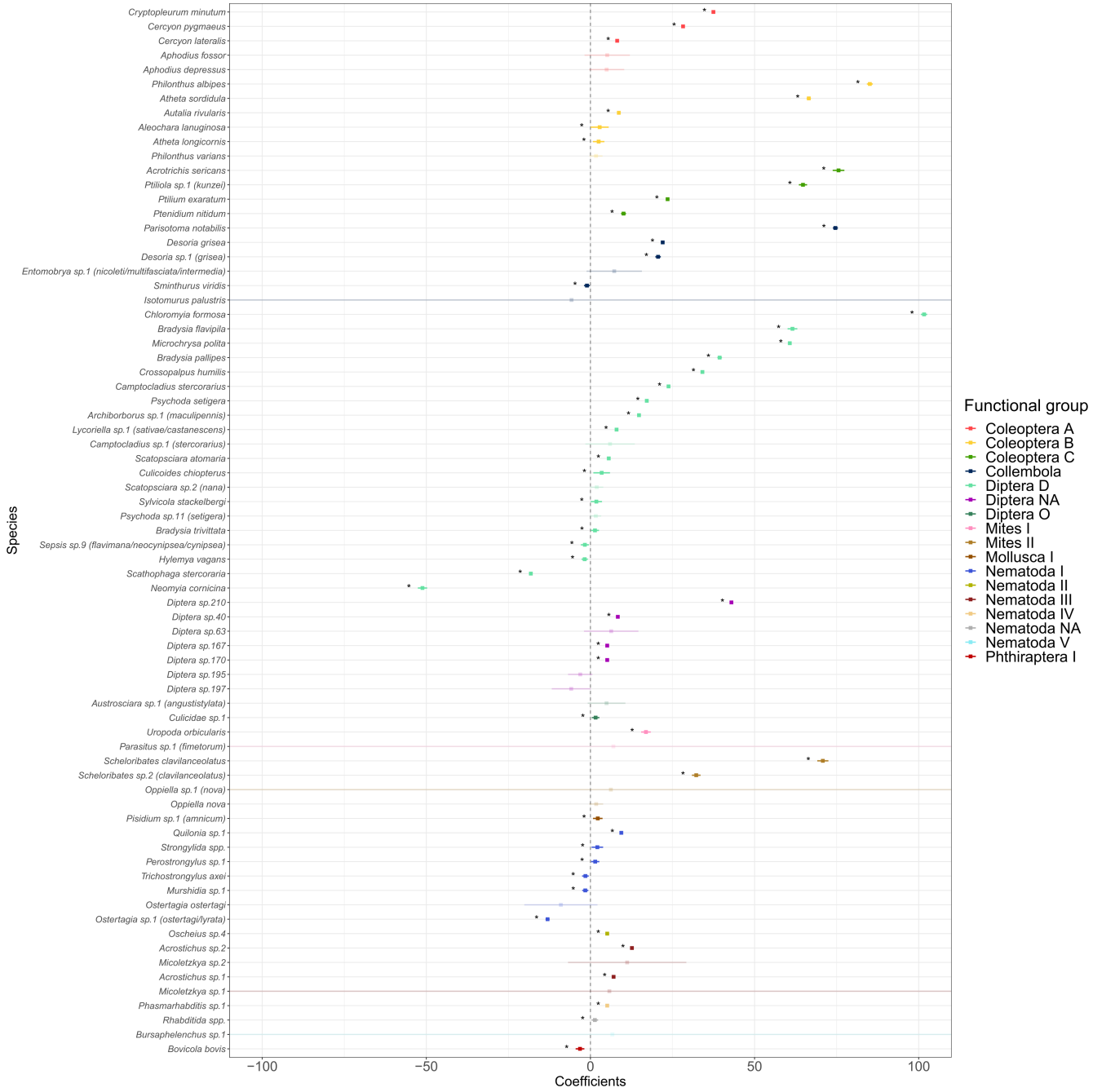

FIGURE 6 Coefficient plot of time (early/late) from the generalized linear latent variable model, including only the species which cumulatively explained >80% of the variance (Chosen by the simper function from the R-package vegan). Each point shows the point estimate for each species, and lines show 95% confidence intervals of the estimate. Species are coloured by functional group, and significant associations with either early or late successional stages are shown with asterisks and bright colours. Note that for a few species (Isotomurus palustris, Parasitus sp.1 (fimetorium), Oppiella sp.1 (nova) & Micoletzkya sp.1), the confidence intervals are very large and continue outside the plot space.

dung via plant material ingested by the cattle (Figure S3, Table S4). A few plant-associated species were detected late in the succession, possibly due to random visits or air-transported DNA (Klepke et al., 2022; Lynggaard et al., 2022). Interestingly, cattle–parasitic nematodes (Nematoda I) showed declining species richness after 11 days (Figure 3), and the external parasitic cattle louse *B. bovis* was no longer detected after 11 days (pasture) and 25 days (meadow), respectively (Figure 3 and Figure S3). DNA from internal parasitic nematodes probably stems from eggs present in the dung throughout the experiment. *Bovicola* species can survive away from the host for up to 2 weeks (Kutz et al., 2012), and thus, we cannot determine whether the detected DNA from *B. bovis* originates from living individuals or from preserved DNA in the dung. However, we consistently detect DNA from this species at the first 2–3 time points, and the presence of live individuals consistently across the 18 dung piles seems unlikely. This indicates that DNA can be preserved in dung for up to 4 weeks after the animal is no longer present.

Our data confirmed the order of appearance of species found by other studies (Gittings & Giller, 1998; Lee & Wall, 2006b; Menéndez & Gutiérrez, 1999; Sladecek et al., 2017; Wassmer, 2020) with a few exceptions. Some beetle species were detected later in the succession in our study compared with previous findings. This was the case for *Aphodius depressus*, *Aphodius rufipes*, *Cercyon haemorrhoidalis*, *C. pygmaeus* and *Cercyon lateralis*. For the two former species, which were rare in the data, these results might be explained by stochastic processes such as PCR bias. But for *C. pygmaeus* and *C. lateralis*, this should not be the case, as they were relatively common in our data. The detection (and field observation) of the fly *M. meridiana* very early in the succession corresponds well with findings by Sladecek et al. (2017) but is much earlier than the 8 days until detection found by Lee and Wall (2006b). These findings support the idea that order of appearance is context-dependent and call for studies investigating succession in cattle dung under different settings.

Patterns of succession did not differ substantially between the two habitat types. However, the initial colonization phase seemed to be somewhat longer in dung piles situated in meadows compared with pasture, as inferred from the greater dissimilarity between t3 and t4 in meadow habitat (Figure 4C). This could be explained by faster desiccation of dung piles in the pasture area, leading to a shorter window of time where the dung is a suitable habitat for colonizing species.

4.2 | Stability of dung-degradation functionality

To preserve dung-degradation as an ecosystem function and thus enhance ecosystem stability, functional diversity with redundancy of species within functional groups of dung-associated fauna is important (Biggs et al., 2020; Milotić et al., 2019). In our study, we found multiple functional groups relevant for dung degradation and associated biotic interactions, and within the two major groups of coprophagous species (Coleoptera A and Diptera D), we consistently detected high species richness after the initial colonization phase (Figure 3). Species from these groups were present throughout the succession, with some arriving early, and others at later stages (Section 3.5, Figures 5 and 6). This could indicate functional stability of dung degradation in this study system, as also found by Slade et al. (2017).

However, because we here treated all coprophagous beetles as the same functional group, we might have missed some functional aspects, which could have been unearthed by, for example, dividing them into endo-, para- and telecoprids (Byk & Piętka, 2018; Milotić et al., 2019). In our study, most species identified in the group Coleoptera A are endocoprids, with only one species representing paracoprids (*Onthophagus similis*). We find it peculiar that we did not detect more paracoprids, such as other *Onthophagus* species or larger scarabs (e.g. *Anoplotrupes stercorosus*, *Geotrupes spiniger* or *Trypocopris vernalis*), which have been frequently registered from the site and were detected with eDNA in a previous study of this site (Thomassen et al., 2023). We expect that the lack of paracoprids in our data represents false negatives due to low abundances, species-specific ecology or primer biases. The latter is a ubiquitous challenge with eDNA metabarcoding, especially when using COI markers (Burian et al., 2021; Deagle et al., 2014).

For other groups (e.g. Hymenoptera H, Diplopoda I and Diptera E & F), species richness was generally low, suggesting that these groups were scarcer in the dung. However, this could also be explained by low database coverage, which is expected to be an issue at least for parasitic wasps, a group containing many undescribed species (Hymenoptera H). It is also *possible* that the amount of eDNA from these groups was simply so low that detection was difficult due to, for example, amplification competition from highly abundant DNA from other species. However, rarefaction curves suggested that our sequencing depth was sufficient (Figure S7). If the low species richness detected in these groups is correct, dung degradation could be affected indirectly if just one or two of these species were to disappear, for example, through parasite release for coprophagous species.

From a functional perspective, much could be gained if measures related to the stage of dung decomposition, such as wetness, gas exchange or dung mass had been included. Several abiotic and biotic factors influence the speed of dung degradation, such as temperature, humidity, precipitation, plant (forage) content and community composition of animals associated with the dung (Floate, 2006; Merritt & Anderson, 1977). Hence, two dung pats sampled at the same time point could potentially be at very different successional stages. In this study, we only used time since deposition and habitat as predictors, and thus, our speculations about functional stability are solely founded in community changes through time after dung deposition. Inclusion of functional measurements could define the successional gradient more precisely and would enable a more direct investigation of the effects of species and functional diversity on dung degradation rates (Stanbrook & King, 2022). Hence, combining eDNA-derived community data with functional measurements in future studies would increase our knowledge about the role of biological communities in decomposition of herbivore dung.

4.3 | Environmental DNA metabarcoding as a method for monitoring succession and dung-associated communities

We found increasing richness of dung-associated invertebrates over time, with peaks in richness occurring at approximately 20–25 days after dung deposition in both meadow and pasture habitats (Figure 2). From a biomonitoring perspective, this means that eDNA sampling should be performed later rather than earlier in the succession, if the goal is to maximize the diversity of detected dung-associated invertebrates. However, as we found large community differences, especially between the early successional stages (Figure 4A,C), sampling several time points along the successional gradient is likely preferable.

We did not find any clear decline in species richness over the time frame of this study (53 days), even though the dung piles were highly degraded at the end of the experiment, and no activity was observed in the piles at the last sampling time point. This might be explained by eDNA being preserved in the dung after the source organism itself has disappeared. Such a lag effect is seen in humus- and mineral-rich substrates such as soil, where DNA is protected from microbial activity by adsorption to humic substrates or minerals (Levy-Booth et al., 2007). In water, DNA degrades fast and is undetectable after approximately 2 weeks (Thomsen et al., 2012), driven at least partly by microbial activity (Zhao et al., 2023). To our knowledge, DNA degradation in dung has not been investigated, but we expect that it might be fast due to the presence of large amounts of bacteria. In fact, the relative read abundances from the cattle parasitic species in this study shows that DNA amounts decrease relatively quickly, despite remaining detectable through longer time frames (Figure S9W,X). This suggests that detection limits could be defined, and used to determine when a species is truly absent from a dung sample. However, as we only used the presence–absence data for the modelling, the preservation of DNA in the dung matrix could explain why fewer of the detected species were clearly associated with early successional stages compared to late stages (Figures 5 and 6). The relative read abundances also revealed several species where proportions of reads were high at the early time points, followed by a decrease and constantly low proportions through the rest of the period (Figure S9). This indicates that such species are associated with early stages of succession, even though it is not revealed from the presence–absence data. Consequently, relative read abundances might serve to distinguish between freshly shed and preserved eDNA and thereby to identify species associated with early succession stages. In addition, more detailed studies of species-specific DNA quantities over time, using qPCR or ddPCR approaches, could increase our knowledge of species-specific activity patterns through succession in dung.

Incompleteness and quality of reference databases can be a major problem when identifying species with DNA-based methods (Goudey et al., 2022; Kvist, 2013). We evaluated the coverage of observed species of arthropods from the study site and found reasonably high coverage at species-level for most orders and families including dung-associated species, except Diptera and Prostigmata (Figure S11). However, at genus-level, coverage was high for all groups meaning that if DNA from species without reference sequences were detected, they would likely match to other species within the genus, and thus appear in the data set as a putative species (e.g. *Sepsis* sp.3 [*duplicata*]). Hence, the diversity estimates from our data might be underestimated at species-level, especially for flies and mites. Consequently, functional redundancy might in fact be even higher within these groups than what we detect. Curation and removal of wrongly identified sequences remain an important problem in public repositories and might influence metabarcoding results (Goudey et al., 2022). In this study, we manually checked blast hits which seemed unreliable, and removed those identified as errors from the taxonomic identification process. Nonetheless, error sequences could result in false-positive or missed detections. These issues remain a methodological caveat, but overall, we do not believe it to have affected the general conclusions drawn.

By using eDNA-based monitoring, not only adults but also cryptic life stages such as larva or eggs can be detected, which is likely overlooked by other methods. However, as it is not currently possible to distinguish between eDNA originating from different life stages, it also represents a limitation, especially when looking at functionality. For instance, the detection of a coprophagous species does not necessarily mean that the species in question is performing any dung degradation functions at the time of sampling. The detected DNA might originate solely from eggs, or dormant life stages, which should be considered when interpreting results from a functional perspective. However, future advances in eRNA analyses might enable the detection of life stages of dung-inhabiting organisms, allowing much better functional inferences from molecular studies of dung communities (e.g. Cristescu, 2019).

5 | CONCLUSIONS

To ensure self-regulating, biodiverse ecosystems, an understanding of the factors relevant for preserving ecosystem functions is needed. Here, we show that eDNA metabarcoding serves as a valuable tool for investigating heterotrophic succession in large mammal herbivore dung. Despite methodological caveats, such as persistence of DNA after species disappearance and incomplete reference databases, eDNA metabarcoding offers several advantages, such as efficient taxonomic identification even in immature life stages, and the possibility to study multiple taxonomic groups simultaneously. We thus expect that studies combining eDNA metabarcoding with measurements of, for example, nutrient contents, moisture levels, dung mass or gas exchange could be highly valuable for elucidating the direct effects of community compositions on ecosystem functions.

Our study demonstrated an example of a system with apparent redundancy of species in the functional groups most important for dung degradation (Coleoptera A, Diptera D). Consequently, dung degrading functionality seemed stable, with an array of species

appearing at either early or late successional stages. We believe that this redundancy of species might serve as a buffering mechanism for decomposition of organic matter in a changing environment. For example, if some species performing the core function of coprophagy are lost, other species performing the same function would likely be ready to replace them and increase in abundance accordingly. We encourage future research to replicate our approach in other systems with different herbivore assemblages and management strategies to draw general conclusions about multi-taxa succession in mammal herbivore dung in terrestrial ecosystems.

AUTHOR CONTRIBUTIONS

Philip Francis Thomsen, Morten D. D. Hansen, Kent Olsen and Eva Egelyng Sigsgaard developed the idea and designed the experiment. Phillip Francis Thomsen and Kent Olsen collected, homogenized and placed dung piles and performed the subsequent sampling. Emil Ellegaard Thomassen and Eva Egelyng Sigsgaard managed the laboratory work. Emil Ellegaard Thomassen performed data analysis, including bioinformatic treatment of sequencing output with inputs from Eva Egelyng Sigsgaard, Mads Reinholdt Jensen and Philip Francis Thomsen. Emil Ellegaard Thomassen wrote the manuscript with inputs from all other authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The raw sequencing data, files for demultiplexing and the output files from MetaBarFlow are available in Dryad here: https://doi.org/10.5061/dryad.zw3r228gj (Thomassen et al., 2024). All other scripts used for data analysis are available upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Species richness through time in functional groups not included in the main figures.

Figure S2. The ordination plots included in the main figures (A and B), and with species MDS scores for all species within several dung-associated group. (C) Coleoptera species, D: Diptera species, (E) Collembola species, (F) Mite species.

Figure S3. A and B: Heatmaps showing occurrences of functional groups (A) and species not colonizing dung (B) in each aggregated sample from pasture plots (left panel) and meadow plots (right panel).

Figure S4. Heatmaps of rare species (occurring in <6 samples) in each aggregated sample.

Figure S5. Accumulated variance between time (early vs. late) samples with each species included from the SIMPER analysis.

Figure S6. Coefficient plot of habitat (meadow/pasture) from the generalized linear latent variable model including the species which accumulatively explained >80% of the variance (Chosen by the

simper function from the *r*-package vegan).

Figure S7. Rarefaction curves for samples from the study. *X* axes show number of reads obtained, and *Y* axes show number of taxa.

Figure S8. Accumulation curves for samples from the study. *X* axes show the number of PCR replicates, and *Y* axes show number of taxa.

Figure S9. Relative Read Abundances (RRA) plots for a selected number of species.

Figure S10. Stacked barplot of each aggregated sample (the three samples from each plot aggregated at each timepoint) from pasture habitat (A) and meadow habitat (B).

Figure S11. Database coverage of observed species from the study site (The Mols Laboratory, 56°13′36″ N, 10°34′33″ E) for all arthropod orders (A-B), all Coleoptera families (C-D) and all Diptera families (E-F).

Table S1. All samples included in the study. Plot refers to the location of the placed dung pad, and dung refers to individual dung pads within each plot.

Table S2. Primers used in this study, PCR settings, including reaction volumes, thermal settings, and number of cycles for the PCR reactions carried out.

Table S3. Reads present in controls (CNEs = extraction blanks, NTCs = PCR blanks).

Table S4. Species present in C_fresh and tExp samples.

Table S5. List of all species identified by eDNA from the dung samples.

Table S6. Coefficients for all species in the generalized linear latent variable model.

Table S7. Adjusted *p*-values for pairwise comparisons of dissimilarity between adjacent sampling points.

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