Testing and optimizing metabarcoding of iDNA from dung beetles to sample mammals in the hyperdiverse Neotropics

Running title: iDNA from dung beetles to sample mammals

Bruno H. Saranholi[1,2*], Filipe M. França[3,4], Alfried P. Vogler[1,5], Jos Barlow[6], Fernando Z. Vaz de Mello[7], Maria E. Maldaner[8], Edrielly Carvalho[9], Carla C. Gestich[2], Benjamin Howes[1], Cristina Banks-Leite[1], Pedro M. Galetti Jr[2]

[1]Department of Life Sciences, Imperial College London, Silwood Park Campus, Ascot SL5 7PY, UK

[2]Departamento de Genética e Evolução, Universidade Federal de São Carlos, São Carlos, SP, Brazil

[3]School of Biological Sciences, Life Sciences Building, University of Bristol, Bristol, BS8 1TQ, UK

[4]Graduate Program in Ecology, Biological Sciences Institute, Federal University of Pará, Belém, 66.075-110, Pará, Brazil

[5]Department of Life Sciences, Natural History Museum, London, SW7 5BD, UK

[6]Lancaster Environment Centre, Lancaster University, Lancaster, UK

[7]Universidade Federal de Mato Grosso, Instituto de Biociências, Departamento de Biologia e Zoologia, Av. Fernando Correa s/n, Boa Esperança, Cuiabá, MT, 78060-900, Brazil

[8]Programa de Pós-Graduação em Ecologia e Conservação da Biodiversidade (PPGECB), Universidade Federal de Mato Grosso (UFMT), Cuiabá 78060-900, Brazil

[9]Programa de Pós-Graduação em Entomologia, Instituto Nacional de Pesquisas da Amazônia, INPA, Manaus 69011-970, Amazonas, Brazil.

*Corresponding author: bruno.saranholi@gmail.com

Testing and optimizing metabarcoding of iDNA from dung beetles to sample mammals in the hyperdiverse Neotropics

Running title: iDNA from dung beetles to sample mammals

Abstract

Over the past few years, insects have been used as samplers of vertebrate diversity by assessing the ingested-derived DNA (iDNA), and dung beetles have been shown to be a good mammal sampler given their broad feeding preference, wide distribution and easy sampling. Here, we tested and optimized the use of iDNA from dung beetles to assess the mammal community by evaluating if some biological and methodological aspects affect the use of dung beetles as mammal species samplers. We collected 403 dung beetles from 60 pitfall traps. iDNA from each dung beetle was sequenced by metabarcoding using two mini-barcodes (12SrRNA and 16SrRNA). We assessed whether dung beetles with different traits related to feeding, nesting, and body size differed in the number of mammal species found in their iDNA. We also tested differences among four killing solutions in preserving the iDNA and compared the effectiveness of each mini barcode to recover mammals. We identified a total of 50 mammal OTUs (operational taxonomic unit), including terrestrial and arboreal species from 10 different orders. We found that at least one mammal-matching sequence was obtained from 70% of the dung beetle specimens. The number of mammal OTUs obtained did not vary with dung beetle traits as well as between the killing solutions. The 16SrRNA mini-barcode recovered a higher number of mammal OTUs than 12SrRNA, although both sets were partly non-overlapping. Thus, the complete mammal diversity may not be achieved by using only one of them. This study refines the methodology for routine assessment of tropical mammal communities via dung beetle 'samplers' and its universal applicability independently of the species traits of local beetle communities.

Keywords: invertebrate-derived DNA, metabarcoding, biodiversity, biomonitoring, Amazonian rain forest.

Introduction

Improving current biodiversity assessments and knowledge is essential to guide international conservation efforts. Vertebrate biodiversity can be assessed through various methods, such as field observations (Keeping and Pelletier, 2014; Varman and Sukumar, 1995), acoustic surveys (Marques et al., 2013) and camera traps (Nichols and Karanth, 2010). While these methodological approaches have improved our understanding of many species' behavior, distribution, and responses to environmental changes (Kiffner et al., 2020; Smith et al., 2020, Marques et al., 2013), they have several limitations, particularly to species with low-density and elusive behavior, as many mammal species (Kinoshita et al., 2019). These traditional methods of surveying species generally require extensive field effort and a high level of taxonomic expertise (Carvalho et al., 2022). These drawbacks have created a demand for alternative techniques to sample biodiversity, particularly within tropical ecosystems, which host most of the global species richness (Barlow et al., 2018), yet are disproportionately under-sampled (Hughes et al., 2012).

Assessing biodiversity through DNA present within environmental samples (i.e., environmental DNA [eDNA]) such as water, soil and snow (Bohmann et al., 2014; Cristescu and Hebert, 2018) has been successful to overcome these limitations of traditional biodiversity monitoring techniques (Kelly et al., 2014; Pikitch et al., 2018). This approach has recently been used to survey several vertebrate taxa (e.g., amphibians, McKee et al., 2015; fish, Olds et al., 2016; reptiles, Kirtane et al., 2019; mammals, Leempoel et al., 2020) and can be more efficient than traditional species survey methods (Carvalho et al., 2022). A more recent approach named iDNA (invertebrate-derived DNA or ingested DNA) has been used for the detection of vertebrates DNA from the gut content of invertebrates (Carvalho et al., 2022 for review), which has become a complementary tool for detecting local mammal communities (Calvignac-Spencer et al., 2013; Gogarten et al., 2019). Mammal biodiversity monitoring can especially benefit from iDNA approaches as many species have elusive behavior and can be rare or present in low population densities, especially within degraded habitats (Ripple et al., 2014). Several groups of invertebrates have been proven to be efficient for sampling vertebrate DNA, such as carrion flies (Calvignac-Spencer et al. 2013, Rodgers et al., 2017; Massey et al., 2021), mosquitoes

(Massey et al., 2021; Saranholi et al., 2023), leeches (Fahmy et al., 2019), and dung beetles (Gillet et al., 2016; Gómez and Kolokotronis, 2016; Drinkwater et al., 2021; Nimalrathna et al., 2023).

Different authors have already used guts or feces to assess mammal DNA, using single-gene PCR (D-loop) to capture horse DNA in guts of different species of dung beetles (Gómez and Kolokotronis 2016), or metabarcoding to investigate diet in adult and larval stage individuals of a flightless dung beetle (*Circellium bacchus*) by comparing the DNA from fecal samples (Kerley et al., 2018), for instance. However, to our knowledge, only three previous studies have adopted iDNA from dung beetles to survey mammal species in the tropics: in African savannas with shotgun sequencing (Gillet et al., 2016), and in Malaysian Borneo rainforest (Drinkwater et al., 2021) and in a Chinese seasonal forest (Nimalrathna et al., 2023) using the metabarcoding protocols. These studies successfully detected some mammal species in the dung beetle iDNA. Nonetheless, it is still unclear whether the dung beetle effectiveness as mammal samplers is affected by their biology, as well as whether methodological features during insect collection, preservation and target DNA to be amplified can impact the iDNA recovered from dung beetles.

Many dung beetles are coprophages, exhibiting trophic relationships with mammals, whilst others are necrophages. In addition to dung consumption, dung beetles also use dung to nest and protect their offspring (Scholtz et al., 2009), being classified into three functional nesting categories. Rollers move dung away from the original dung pad, tunnellers excavate tunnels closely beneath dung pads, while dwellers live on the dung pad itself (Tonelli, 2021). Given their different feeding habit (coprophagous and necrophagous), nesting behavior (roller, tunneller and dweller), easy and cost-effective sampling (Nichols and Gardner, 2011; Gardner et al., 2008), dung beetles can provide a good representation of mammalian biodiversity. Although coprophagous dung beetles may visit a higher number of diet resources (Frank et al., 2018), if they are more efficient as iDNA samplers have not been evaluated thus far. On the other hand, body size would improve the chances of mammal DNA detection, by ingesting higher volumes of dung (Gómez and Kolokotronis, 2016), while, among nesting behavior, rollers may identify a minor number of mammals, tending to rely on a single dung source (Nimalrathna et al., 2023). Dung beetle surveys carried out with distinct ecological purposes commonly use different killing

solutions to preserve the dung beetle bodies (e.g. Aristophanous, 2010; Mora-Aguilar et al., 2023), but as far as we know, no previous study evaluated its efficiency for iDNA studies. Also, previous studies in the neotropical region indicated that the combination of two mini-barcodes (e.g., 12SrRNA and 16SrRNA) could provide broader representativeness of the mammal diversity detected in carrion-fly and mosquito iDNA (Rodgers et al., 2017; Saranholi et al., 2023), but this pattern was not tested with dung beetles.

Here, we tested the effectiveness of iDNA for sampling a broad range of hyperdiverse mammal fauna and evaluated if some biological and methodological aspects affect the use of dung beetles as mammal species samplers. To achieve this, we sampled dung beetles with pitfall traps in the Brazilian Amazonia and evaluated the dung beetles as samplers of the local mammal biodiversity using iDNA metabarcoding. We obtained the number of mammal species that can be detected in a single dung beetle individual, and tested if distinct dung beetle nesting behavior, feeding strategies, and body size can affect mammals sampled through iDNA. We also evaluated differences among killing solutions in preserving the DNA, by evaluating the number of reads recovered from each detected mammal through dung beetle iDNA. Finally, we compared the effectiveness of two mini-barcodes to identify mammals from dung beetle iDNA within a highly biodiverse tropical forest. For that, we contrasted the number of OTUs and reads of mammals detected and the exclusive mammal species identified by each mini-barcode.

Material and Methods

Study area and insect sampling

We collected dung beetles in March 2019, within the Tapajós National Forest (FLONA-Tapajós; Fig. 1), a Brazilian protected forest of 527,319 ha within the Amazon biome (Carvalho et al. 2023), with around 4,000 people from local communities living in it (ICMBio, 2019). We used 48 dung-baited traps distributed in six 700-m transects (8 traps/transect) placed at least 4,000 meters from each other. Most traps (n = 36) consisted of 1-L plastic containers (19 cm diameter and 11 cm deep) containing approximately 200 mL of a killing solution (water and salt, alcohol, or ethylene-glycol). To evaluate differences in the iDNA preservation between killing solutions, we also collected dung beetles with no-killing funnel pitfall traps (n = 12) made from

2-L clear plastic bottles adapted to prevent beetles from escaping (10 cm diameter and 20 cm deep; Fig. S1). We cut off the complete tapering part and the small neck of the bottle top, forming a funnel. This funnel was placed upside down into the bottom of the bottle. Around 8-10 punctures were made with a needle in the bottom part to prevent it from flooding and soil was added to it when placing traps in the field (Fig. S1). All pitfall and funnel traps were buried with their opening at ground level and had a suspended bait container with a mixture of pig (*Sus scrofa*) and human feces (4:1 pig to human ratio; as in previous studies (Marsh et al., 2013; Carvalho et al., 2023), which was protected from dung beetles by a fine netting. Each transect had two traps with each killing solution (n = 6) and two funnel traps, all separated by 100 meters (Fig. 1). After 48h, all traps were examined and the dung beetles were collected, preserved in 96% ethanol, and stored at -20 °C until laboratory procedures.

All sample collections were conducted in accordance with Brazilian legislation and under the appropriate permits: SISBIO—Sistema de Autorização e Informação em Biodiversidade – MMA/ICMBIO (53271-9) and National System of Genetic Resource Management and Associated Traditional Knowledge (SisGen A9F8717).

Morphological identification and gut extraction

We analyzed a total of 403 dung beetles. All individuals were identified at the species level whenever possible according to recent taxonomic revisions and morphological comparison with the reference collection from the “Entomological Section of the Zoological Collection in the Federal University of Mato Grosso" (CEMT, https://collectory.sibbr.gov.br/collectory/public/show/dr435). A total of 37 different dung beetle species were identified (Supplementary Table S1). Based on the species identification, dung beetles were divided into three groups according to their feeding habit (coprophagous and necrophagous), nesting behavior (roller, tunneller and dweller), and species size (small, medium, and large) (Supplementary Table S1). The gut from each dung beetle individual was dissected from the abdominal cavity using sterilized forceps and a stereomicroscope; and stored in 96% ethanol at -20 °C until iDNA extraction. All collected dung beetles are deposited in the CEMT dung beetle collection (Supplementary Table S1), Brazil.

DNA extraction, Mini-barcode amplification, and Metabarcoding sequencing

The iDNA from the gut of each dung beetle individual was extracted separately using DNeasy Blood & Tissue Kit (Qiagen®) following the manufacture protocol, in an iDNA-dedicated laboratory, including negative controls. The obtained DNA was eluted in 100µl of elution buffer. For each specimen, mitochondrial 12SrRNA and 16SrRNA rRNA genes were amplified using the primers 12SV5F and 12SV5R (Riaz et al., 2011) and 16Smam1 and 16Smam2 (Taylor, 1996) to produce amplicons of approximately 130-140 bp. These mini-barcodes are commonly used in iDNA studies for mammal community detection (Rodgers et al., 2017; Lynggaard et al., 2019; Massey et al., 2021; Saranholi et al., 2023), and identified reference sequences of Amazonian mammals are available for both genes, mainly for the 12SrRNA mini-barcode used (Kocher et al., 2017). The 12SV5F primer was made degenerate at the first base (5´ - YAGAACAGGCTCCTCTAG - 3´), to broaden its taxonomic range (Kocher et al., 2017). Unique identifiers (*tags*) obtained from Axtner et al. (2019) were added to both forward and reverse primers to label each PCR amplicon (Supplementary Table S2), allowing to obtain the individual information of each dung beetle. The PCR protocols for both mini-barcodes followed Rodgers et al. (2017), with minor modifications: 1x buffer (Tris–HCl 20 mM pH 8.4 and KCl 50 mM), 0.4 mM of each primer, 0.2 mM dNTP (Invitrogen), 4 and 2 mM $MgCl_2$ for 16SrRNA and 12SrRNA, respectively, 1.5 U Platinum Taq polymerase (Invitrogen), and 3 µl of template DNA. Cycling conditions were 10 min at 95°C, followed by 42 cycles of 30 s at 95°C, 30 s at 64°C and 50°C for 16S rRNA and 12S rRNA, respectively, and 1 min at 72°C, with a final extension of 10 min at 72°C. All DNA extractions and PCRs were prepared in dedicated rooms within a UV-sterilized hood, sanitized with 2% bleach solution before each new procedure. We also included a no-template DNA as negative control to check for contamination. PCR amplification success was checked in 1.5% agarose gel. A second PCR amplification was conducted for the DNA samples that failed in the first attempt following the same conditions of the first reaction. The PCR products of each individual for both mini-barcodes were aliquoted, and these aliquots were pooled into 54 samples for large-scale sequencing (Supplementary Table S2). PCR negative controls were also included for large-scale sequencing.

For the metabarcoding sequencing, the pools were cleaned using magnetic beads (1.2µl Agencourt AMPure XP® Beckman Coulter per 1µl PCR product), quantified in a Qubit fluorimeter (Thermo Fisher), normalized to 50ng/µl, and indexed using the Nextera Index kit® (Illumina). The paired-end metabarcoding sequencing was performed in two runs, processed in the Illumina iSeq® equipment, using the iSeq 100 v2 300 (2x150 bp) cycles reagent kit, for a total of 70,000 to 100,000 reads per metabarcoding sequencing sample (Supplementary Table S2).

Bioinformatic, Sequence analysis and taxonomic identification assignment

The resulting sequences were demultiplexed using *process_radtags* in Stacks v2.59 (Catchen et al., 2013), in which the identifier barcodes (*tags*) were used to trace back the information to each individual (Axtner et al., 2019). At this step, the barcode option - -inline-inline was used to eliminate misassignments caused by occasional tag-jumping events, which could result in incorrect matching forward and reverse tag sequences (Schnell et al., 2015; Axtner et al., 2019). For the reads obtained from each dung beetle individual, we used PEAR v.0.9.11 (Zhang et al., 2014) to merge the correspondent forward and reverse sequences and trimmed them to a minimum quality score threshold (-q) of 15, a minimum overlap (-v) of 100 base pairs, and minimum length (-n) of 100 base pairs. Then, we separated the 12SrRNA and 16SrRNA sequences, by setting 20% of the maximum mismatch within the primer region, and removed primer sequences with the *cutadapt* function (Martin, 2011). After these steps, we performed clustering of OTUs (Operational Taxonomic Units) for the reads of each mini-barcode separately for each tagged sample using USEARCH (Edgar, 2010), considering 97% of similarity among sequences and discarded all singletons from the analysis. We also discarded OTUs with relative abundance lower than 0.05% reads within each sequenced pool (corresponding to 14 – 38 reads for a given pool of metabarcoding sequencing). The final OTUs sequences were identified against GenBank (https://www.ncbi.nlm.nih.gov/genbank/) for vertebrate species identification. Species-level assignments followed the criteria of high percentage of matches (at >98% nucleotide similarity). When a sequence had a match for two or more species, we assigned the species identity according to the expected species occurrence in the study area based on the information available in the IUCN (International Union for Conservation of Nature) and GBIF (Global Biodiversity Information Facility) databases and information from local

mammal species surveys (Brocardo et al., 2022; Ravetta and Brocardo, 2022). Similarity matches to species not previously recorded from the study area were assigned to a close relative from the same genus with known occurrence in the region. Where high-similarity matches were obtained to more than one species from one genus, only the genus level was assigned. Sequences with <98% similarity to a Genbank entry were assigned to genus, family, or order level only, and sequences with less than <90% similarity were not assigned taxonomically, as commonly used in iDNA studies (Rodgers et al., 2017; Massey et al., 2021). Negative controls of both the DNA extraction and PCR only detected human and *S. scrofa* sequences, which were excluded from the analyses.

Data Analysis

First, to characterize the mammal detection success according to the sampling effort and the used mini-barcodes, we performed an accumulation curve, based on a permutation (10,000 permutations) procedure and random method (Gotelli and Colwell, 2001) using *specaccum* function from vegan package (Oksanen et al., 2013).

We used a generalized linear model (GLM) to assess whether the traits of the dung beetles (size, nesting strategy, feeding habit), as well as the type of killing solution (non-killing, water salt, ethylene glycol, ethanol solutions) or mini-barcode used (12SrRNA, 16SrRNA) influenced the number of mammal OTUs obtained per specimen. We used a poisson distribution where the count of the unique mammal OTUs obtained per specimen was our response variable, and beetle size, beetle nesting strategy, beetle feeding strategy, killing solution, and mini-barcode were explanatory variables. We also included random intercepts for the dung beetle species and transects to remove such effects and ensure that results were not being primarily driven by these factors.

Finally, we used a GLM to assess whether the type of killing solution or mini-barcode used influenced the number of mammal reads obtained. The fitted model used a negative binomial distribution and a zero-inflation term to account for overdispersion and zero-inflation in the residuals. The model also includes an offset for the total number of reads achieved per

metabarcoding run after bioinformatic filtering to ensure the observed effects were not simply an artifact of successful metabarcoding runs.

All GLMs were run using the glmmTMB package (Brooks et al., 2017) in R v.4.2.1 environment (R Core Team, 2022), whilst assumptions were tested using the DHARMa package (Hartig, 2022). Random effects were chosen in accordance with sampling design rather than model selection criteria; though the OTU model used had a lower AIC than the same model without random effects. We used the contrast and comparisons functions from the emmeans package (Lenth, 2023) to identify significant differences between levels of our explanatory variables at the 95% confidence interval level.

Results

From an initial total of 3,215,211 paired sequence reads, we retained 243,198 reads assigned to mammals, with a mean of 574 ± 930 (SD) mammal reads per successfully amplified beetle specimen (12SrRNA: 337 ± 620 SD, N=148; 16SrRNA: 718 ± 1051 SD, N=243), totaling 47 OTUs of wild mammal species, from ten orders. From that, we assigned 32 OTUs at the species level, eleven at genus, and eight at the order level (Table 1). In addition, 13 non-mammal OTUs were detected only when using the 12SrRNA mini-barcode (birds: pigeons - *Columba* sp., eared dove - *Zenaida auriculata*, Southern mealy amazon - *Amazona farinosa*, short-tailed parrot - *Graydidascalus brachyurus*, house sparrow - *Passer domesticus*, white-throated tinamou - *Tinamus guttatus*, antshrike - *Thamnophilus* sp., dove - *Geotrygon* sp., woodpecker - *Veniliornis* sp., dark-winged trumpeter - *Psophia viridis*; and amphibia: frog - *Leptodactylus* sp., tropical bullfrog - *Adenomera* sp., *Pristimantis* sp.), although in a smaller number of individual dung beetles (Supplementary Table S3).

Mammal species accumulation curves indicated that the number of new mammal species detected decelerated beyond a sample count of 150 beetle specimens (Fig. 2). Some mammal OTUs were recovered from multiple dung beetle individuals and were present in up to 73 beetle samples, such as crab-eating fox (*Cerdocyon thous*) and Brazilian porcupine (*Coendou prehensilis*) (Table 1). Still, many mammal species were detected in only one dung beetle individual – e.g., jaguarundi (*Herpailurus yagouaroundi*), bush dog (*Speothos venaticus*), kinkajou (*Potos flavus*),

raccoon (*Procyon cancrivoros*), Southern naked-tailed armadillo (*Cabassous unicinctus*), opossum (*Marmosops sp.*), woolly mouse opossum (*Micoureus demerarae*), giant anteater (*Myrmecophaga tridactyla*), Southern tamandua (*Tamandua tetradactyla*), black-capped capuchin (*Sapajus apella*), and tuff-tailed spiny tree rat (*Lonchothrix emiliae*) (Table 1). At least one mammal species was detected in 70% of the 403 dung beetles analyzed. The mean number of species detected in each dung beetle DNA extract was 1.59 ± 1.51 (range: 0 – 7). The number of OTUs obtained per dung beetle did not differ according to beetle size, nesting or feeding strategies (Fig. 3). There was also no significant difference in the OTU numbers obtained per beetle due to differences in killing solution used. However, there was a significant difference in numbers of mammal species detected with 12SrRNA and 16SrRNA (ratio = 0.45, 95% CI = [0.384, 0.533]; Fig. 3), with 16SrRNA retrieving 40% more OTUs than 12SrRNA. Yet, 8 and 20 mammal OTUs were exclusively obtained by 12SrRNA and 16SrRNA, respectively (Table 1). Consequently, the rate of accumulation of mammal species was much higher for both mini-barcodes combined than for each marker separately (Fig. 2).

In general, 16SrRNA recovered more mammal reads than 12SrRNA (ratio = 0.55, 95% CI = [0.436, 0.694]; Fig. 4A). The number of mammal reads detected by dung beetle iDNA significantly differed between specimens killed using ethanol and ethylene (ratio = 0.42, 95% CI = (0.246 , 0.718)), as well as ethanol and water (ratio = 0.43, 95% CI = (0.248, 0.727)), where significantly fewer reads were obtained from specimens killed using ethanol than those using water or ethylene, but did not differ between any other pairs of killing solutions (Fig. 4B).

Discussion

Our study shows that iDNA from dung beetles associated with metabarcoding is suitable for detecting many tropical forest mammals. We successfully assessed a large representation of the mammal community from Tapajós National Forest, as suggested by the species accumulation curve (Fig. 2), totaling 47 native mammal OTUs from ten orders. Considering only the species-level assignments (N = 32), our iDNA survey recovered about 70% of the terrestrial medium and large non-primate mammals (100% of Perissodactyla and Myrmecophagidae, 75% Cingulata, 60% Rodentia, 57% Carnivora and 25% Artiodactyla), and 54% of primates previously

recorded within FLONA-Tapajós (Brocardo et al., 2022; Ravetta and Brocardo, 2022). Some species (e.g., *Cerdocyon thous*, *Pecari tajacu*, *Dasypus* sp., and *Coendou* sp.) were more frequently registered than others, potentially reflecting their higher abundance, as noted in previous studies (Gillet et al., 2016; Drinkwater et al., 2021). Our study also revealed rare terrestrial or arboreal mammal species that are not easily detected using traditional survey methods (e.g., *Priodontes maximus, Speothos venaticus*, *Potos flavus*), highlighting the potential of dung beetle iDNA to sample these more elusive mammals.

Three domestic mammal species were also identified (Table 1). Similar results were found by Massey et al. (2021), who also identified the same domestic species in the iDNA of flies, sand flies, and mosquitoes in the Brazilian savanna ecotone. The presence of local communities living in the FLONA - Tapajós forest reserve (ICMBio, 2019) may explain the detection of these species in the iDNA, possibly entering the forest via alternative pathways, such as ingestion and defecation from humans or mobile predators. Even though we have found no evidence of contamination in our negative controls, this result may not provide unambiguous proof of no contamination, as these species are often considered contaminants in metabarcoding (e.g. Champlot et al. 2010) and some caution while investigating the true meaning of their detection is still necessary.

The detection of non-mammals among the recovered OTUs reinforce the suggestion that some dung beetles also utilize other vertebrates in Amazonia (e.g., Correa et al., 2023; Carvalho et al., 2023). Feeding on dung and carrions of birds has previously been recorded in an avian-dominated island where mammals were depleted (Stavert et al., 2014). Our results suggest the behavior can also be found in regions with rich mammal faunas, broadening our understanding of the resources used by dung beetles. Scarabaeinae dung beetles are well known for necrophagy in particular in the Neotropics, e.g. in the spectacular large beetles of the genus *Coprophaeneus*, which bury carcasses for breeding. The detection of bird and amphibian OTUs thus may reflect the consumption of small carcasses. Clearly the primers used here are not appropriate to detect a broad range of vertebrates, which revealed this poorly recorded feeding source of dung beetles. But, even with the current strategy this finding greatly extends the utility of dung beetles as ‘samplers’ of a much wider range of potentially rare or elusive

316 vertebrate groups. Further investigation is also required to understand if dung beetles are using
317 carrion or feces from these non-mammal species, and if certain dung beetles are specialists.
318 No statistically significant differences were found in the number of OTUs obtained by dung
319 beetles of different feeding and nesting behavior, body size and taxonomic affiliation. These
320 findings support the universal applicability of dung beetles for sampling of vertebrate
321 communities without bias from differences in morphological and functional traits. Yet, we
322 recommend further investigation to better explore the composition of mammal species in the
323 diet of distinct dung beetle groups. For instance, Frank et al. (2018) showed that coprophagous
324 dung beetles may visit a high number of diet resources, which may result in a diversity of
325 mammal species in their iDNA (Gillet et al., 2016; Drinkwater et al., 2021; Nimalrathna et al.,
326 2023). The absence of difference in the number of mammal OTUs detected here by
327 coprophagous and necrophagous dung beetles, suggests that both groups are able to assess the
328 diversity of mammals. The similarity of the two groups could also represent preferences rather
329 than strict associations, and the high level of plasticity in this trait which allows species ability to
330 adapt their diets depending on resource availability (e.g., Salomão et al. 2018). Thus, the
331 classification of feeding style is not definitive, as many species switch between coprophagy and
332 necrophagy, in part driven by seasonal changes in substrate quality in dry and wet periods (e.g.,
333 Cambefort, 1991; Medina and Lopes, 2014).
334 The diversity of mammals obtained here was also similar among the different dung beetle
335 nesting behaviors studied. These findings contrast with previous research showing that
336 tunnellers detected a higher number of mammal hosts (Nimalrathna et al. 2023). These
337 contrasting results are probably a consequence of differences in the sampling effort.
338 Nimalrathna et al. (2023) analyzed 18 dung beetle specimens from three species (only one
339 tunneller: *Onthopagus diabolicus*), while our results are based on 37 species and a larger
340 sampling (229 tunnellers, 91 roller and 83 dweller specimens), which may provide better
341 representativeness of the diversity of dung beetles and nesting behaviors. Rather than allowing
342 conclusions about the feeding style, iDNA is unequivocal about the identity of the host species
343 and as such will enhance our understanding of dung beetle-mammal interaction networks and

how they might respond to environmental changes in the tropics (Chiew et al. 2021; Raine et al. 2018).

Up to seven mammal OTUs were recovered in a single dung beetle, which may be explained by the capability of metabarcoding for detection of small amounts of iDNA deriving from different feeding events. This higher number of OTUs occurred in rare events (only four cases within our whole data) and the mean number of mammal OTUs per dung beetle individual (1.5 ± 1.5) was lower. Although most iDNA study did not show insect individual results (Rodgers et al., 2017; Lynggaard et al., 2019; Gogarten et al., 2020; Massey et al., 2021), the mean detection of mammal species per individual here was similar to those previously reported in dung beetles (Nimalrathna et al., 2023) and in flies (Calvignac-Spencer et al., 2013). Although Saranholi et al. (2023) reported higher mean values in mosquitoes (3.6 ± 4.3 OTUs) and carrion flies (saprophagous flies: 2.7 ± 1.7 OTUs, hematophagous flies: 2.8 ± 1.4 OTUs), it is important to note that these latter study was conducted in a Zoo with several confined animals, which likely increased the number of mammal species encountered by each individual.

We found no differences in the number of mammal OTUs associated with dung beetle size. It might be expected that body size is correlated with successful detection of mammalian DNA because larger dung beetles consume more dung (Gómez and Kolokotronis, 2016). However, other factors such as the time beetles spent feeding before capture and for dung digestion since the last feeding might influence mammal detection (Gómez and Kolokotronis 2016; Drinkwater et al., 2021).

On the level of the entire mammal community, the number of OTUs obtained in our study was higher than in previous research using iDNA from dung beetles. Drinkwater et al. (2021) detected only six wild mammal species obtained with one-primer pair (16SrRNA) iDNA metabarcoding of at least 300 dung beetles captured during one-day collection using 108 pitfall traps in Borneo. In turn, Gillet et al. (2016) detected seven mammals in Essuatini (Africa), using iDNA shotgun sequencing of 11 dung beetles captured in two-day passive collection, with two flight interception traps. The higher number of mammal species detected in our study could be explained by difference in the sampling design and intensity, such as our higher number of traps (N = 48), longer trap exposure in the field (N = 48h), broader spatial distribution of traps and

transects (N = six 700-m transects separated by at least 4 km), and the greater number of specimens tested (N = 403), which increased the chance of sampling more beetle and diet diversity. In addition, while the 16SrRNA mini-barcode was more effective for mammal species detection, the total species number increased by using the 12SrRNA barcode (Fig. 2). Similar trends were also reported when both primer pairs were used to amplify iDNA obtained from mosquitoes and carrion flies (Saranholi et al., 2023), suggesting that combining both markers may provide a fuller representation of the targeted biodiversity. In addition, the high richness of the Amazonian mammal community present in the study area, which is expected to hold around 35 large and medium size terrestrial mammals (Brocardo et al., 2022), and 13 species of arboreal primates (Ravetta and Brocardo, 2022), can also explain the high number of mammals detected here.

Of the OTUs detected in our study, 68% were assigned at the species level, as identified either by the 12SrRNA or 16SrRNA markers, or both. This value is higher than that obtained by Drinkwater et al. (2021) using only 16SrRNA and dung beetle iDNA (50%), and higher (40% and 45%, Lynggard et al., 2019) and similar (66%, Massey et al., 2021) to those based on iDNA from samplers of other insect groups. The number of OTUs identified at species level is highly dependent on the completeness of the reference sequence dataset, and altogether these results indicate that the representativeness of the reference library is still a challenge, mainly in the hyperdiverse tropics. In our study, we noticed that even using two mini-barcodes to improve the species-level assignments, the lack of reference sequences for all mammals inhabiting the study area led us to use the species distribution information to confirm the correct OTU assignment in 13 cases (Table 1), representing 28% of the native mammal OTUs detected. Rodgers et al. (2017), using iDNA from carrion flies to survey mammals on a tropical island, assigned 60% of the OTUs at the species level, although when taking into account information about local species occurrences, an assignment to species was achieved in 100% of samples. Occurrence records need to be at the species level, and thus , our study reinforces the urgent need for enhancing reference sequences available in global library databases.

We found here no effect of the different trapping approaches on the iDNA quality, represented by the number of OTUs obtained from dung beetles, although the number of reads

differed between the killing solutions. Surprisingly, using water and salt to kill and keep dung beetles for the first 48h had better results than ethanol in the number of reads obtained. Considering that all dung beetles were preserved in ethanol after 48h, we are not aware of any plausible explanation for ethanol not being a good killing solution. Despite differences in the number of reads obtained from different killing solutions, the total number of OTUs identified did not differ, suggesting that all solutions are equally good at inferring species presence and associated metrics such as richness.

It should be noted that the paired-end reads which matched with human and *S. scrofa* and were discarded from the analysis, were likely due to contamination by human handling and the sampling bait used, respectively, despite the care during trap manipulation and placement. Indeed, Massey et al. (2021) found that 80% of the total sequences of the iDNA from mosquitoes were from humans, which were assumed as contamination. As such, it appears that iDNA methods can still be made more efficient, through improved laboratory methods and alternative practices of sample acquisition such as the use of unbaited traps including flight interception traps. We also suggest the use of blocking primers for human and pig, which could reduce contamination by human handling and from the bait used in the pitfall traps, respectively. Utilizing blocking primers for non-target species might increase both the quantity and diversity of DNA detected, thereby enhancing the detection of wild species, particularly rare ones (Boessenkool et al., 2012). However, concerns about blocking primer specificity and concentration used should always be considered to avoid inhibiting DNA amplification, if primates and artiodactyla are target.

In summary, we found that our metabarcoding of the iDNA of dung beetles was able to sample many of the non-volant mammal species inhabiting Tapajós National Forest, including rare species, highlighting the potential of iDNA from dung beetles to sample elusive mammals. No interrelationship between number of mammal OTUs and the dung beetles feeding and nesting behavior, body size and taxonomic affiliation was observed. Therefore, neither 16SrRNA or 12SrRNA alone successfully detected all mammal OTUs observed, and we recommend the use of both primer pairs for metabarcoding, which appears to be essential to a more secure detection and identification of a broader representation of the mammal community, especially

in the hyperdiverse areas. The reliable detection of target vertebrate groups by using iDNA from dung beetles provides a powerful tool for mammal survey and monitoring worldwide.

AUTHOR CONTRIBUTIONS

BHS, FMF, APV, FZVM, JB, CBL and PMGJ conceptualized and performed the study design. FMF conducted fieldwork. MEM and EC conducted beetle sorting out and species identification. BHS performed the laboratory activities and led data analysis with inputs from FMF, APV, JB, FZVM, CBL and PMGJ. The first draft of the manuscript was written by BHS, FMF, CBL and PMGJ. All authors contributed to discussing the results and revising the manuscript.

ACKNOWLEDGEMENTS

This study was supported by the Natural Environment Research Council (NERC, NE/S011811/1). BHS received NERC (NE/S011811/1) fellowship. PMGJ thanks Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, 303524/2019-7). FF acknowledges funds and support provided by the UK Research and Innovation Future leader Fellowship (Medical Research Council [grant number MR/X032949/1]); CNPq (CNPq-INCT 406767/2022-0 [INCT/SinBiAm], CNPq-PPBio 441257/2023-2 [PPBio-AmOr], CNPq-CAPES 441659/2016-0 and 441573/2020-7 [PELD-RAS], and MCIC-CNPq 420254/2018-8 [RESFLORA]); the University of Bristol via Liv Sidse Jansen Memorial Foundation (FOR-TRAITS; ID: 1777136), PolicyBristol (SYNPAM; ID: 1989427), Cabot Seedcorn 2023 (Voices of Amazonia; ID: 2258319), and the Climate and Net Zero Impact Awards (Scaling-up TAOCA, ID: 170839). FF, PG and JB also acknowledge support by the Climate and Biodiversity Initiative of the BNP Paribas Foundation (Project BIOCLIMATE). The authors are also thankful for the constructive comments of the anonymous reviewers, which improved the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests

DATA AVAILABILITY STATEMENT

Raw sequence data are available in the NCBI BioProject and in the Sequence Read Archive repository under Accession Number PRJNA1075326. Dung beetle community data (species and abundances) are available through the TAOCA biodiversity database (https://www.taoca.net/).

BENEFIT-SHARING STATEMENT

Benefits from this research accrue from the sharing of our data and results on public databases as described above.

SUPPORTING INFORMATION

Additional information is available in the electronic supplementary material.

References

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data.

Aristophanous, M. (2010). Does your preservative preserve? A comparison of the efficacy of some pitfall trap solutions in preserving the internal reproductive organs of dung beetles. *ZooKeys*, 34, 1-16.

Axtner, J., Crampton-Platt, A., Hörig, L. A., Mohamed, A., Xu, C. C., Yu, D. W., & Wilting, A. (2019). An efficient and robust laboratory workflow and tetrapod database for larger scale environmental DNA studies. *GigaScience*, *8*(4), giz029.

Barlow, J., França, F., Gardner, T. A., Hicks, C. C., Lennox, G. D., Berenguer, E., ... & Graham, N. A. (2018). The future of hyperdiverse tropical ecosystems. Nature, 559(7715), 517-526.

Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., ... & De Bruyn, M. (2014). Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in ecology & evolution*, *29*(6), 358-367.

Brocardo, C. B., Rosa, C. A., Sampaio, R., Castro, A. B., Rossi, L. C., Rosa, D. P., Nóbrega, J., Fadini, R. F. (2022) Mamíferos de médio e grande porte (exceto primatas) da Floresta Nacional do Tapajós e da Reserva Extrativista Tapajós-Arapiuns. In: Brocardo, C. R., Giacomin, L. L.

(Org). *Biodiversidade na Floresta Nacional do Tapajós e na Reserva Extrativista Tapajós – Arapiuns*. Santarém, Pará: UFOPA, 2022.

Brooks, M. E., Kristensen, K., van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., Skaug, H. J., Maechler, M., Bolker, B. M. (2017). "glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling." *The R Journal*, 9(2), 378–400. doi:10.32614/RJ-2017-066.

Calvignac-Spencer, S., Leendertz, F. H., Gilbert, M. T. P., & Schubert, G. (2013). An invertebrate stomach's view on vertebrate ecology: Certain invertebrates could be used as "vertebrate samplers" and deliver DNA-based information on many aspects of vertebrate ecology. *BioEssays*, *35*(11), 1004-1013.

Calvignac-Spencer, S., Merkel, K., Kutzner, N., Kühl, H., Boesch, C., Kappeler, P. M., ... & Leendertz, F. H. (2013). Carrion fly-derived DNA as a tool for comprehensive and cost-effective assessment of mammalian biodiversity. *Molecular ecology*, *22*(4), 915-924.

Cambefort Y. (1991). Dung beetles in tropical savannas. In: Cambefort Y, Hanski I (eds) *Dung beetle ecology*. Princeton University Press, Princeton, pp 156–178.

Carvalho, E. C., Maldaner, M., Costa-Silva, V., Sehn, H., Franquini, C., Campos, V., ... & França, F. (2023). Dung beetles from two sustainable-use protected forests in the Brazilian Amazon. *Biodiversity Data Journal*, 11, e96101.

Carvalho, C. S., De Oliveira, M. E., Rodriguez-Castro, K. G., Saranholi, B. H., & Galetti Jr, P. M. (2022). Efficiency of eDNA and iDNA in assessing vertebrate diversity and its abundance. *Molecular ecology resources*, *22*(4), 1262-1273.

Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., & Cresko, W. A. (2013). Stacks: an analysis tool set for population genomics. *Molecular ecology*, 22(11), 3124-3140. https://doi.org/10.1111/mec.12354.

Champlot, S., Berthelot, C., Pruvost, M., Bennett, E. A., Grange, T., & Geigl, E. M. (2010). An efficient multistrategy DNA decontamination procedure of PCR reagents for hypersensitive PCR applications. *PLoS One*, *5*(9), e13042.

513 Chiew, L. Y., Hackett, T. D., Brodie, J. F., Teoh, S. W., Burslem, D. F., Reynolds, G., ... & Slade, E.
514 M. (2022). Tropical forest dung beetle–mammal dung interaction networks remain similar
515 across an environmental disturbance gradient. *Journal of Animal Ecology*, 91(3), 604-617.
516 Correa, C. M., Salomão, R. P., Xavier, B. F., Puker, A., & Ferreira, K. R. (2023). Not all dung
517 beetles feed on dung: Scarabaeinae (Coleoptera: Scarabaeidae) attracted to different
518 carrion types in contrasting habitats at Brazilian Amazon. *Austral Ecology*, 48, 952–968.
519 Cristescu, M. E., & Hebert, P. D. (2018). Uses and misuses of environmental DNA in biodiversity
520 science and conservation. *Annual Review of Ecology, Evolution, and Systematics*, 49, 209-
521 230.
522 Drinkwater, R., Williamson, J., Clare, E. L., Chung, A. Y., Rossiter, S. J., & Slade, E. (2021). Dung
523 beetles as samplers of mammals in Malaysian Borneo—a test of high throughput
524 metabarcoding of iDNA. *PeerJ*, *9*, e11897.
525 Edgar, R. (2010). *Usearch*. Lawrence Berkeley National Lab. (LBNL), Berkeley, CA (United States).
526 Fahmy, M., Ravelomanantsoa, N. A. F., Youssef, S., Hekkala, E., & Siddall, M. (2019). Biological
527 inventory of Ranomafana National Park tetrapods using leech-derived iDNA. *European*
528 *Journal of Wildlife Research*, *65*(5), 1-13.
529 Frank, K., Krell, F. T., Slade, E. M., Raine, E. H., Chiew, L. Y., Schmitt, T., ... & Blüthgen, N. (2018).
530 Global dung webs: high trophic generalism of dung beetles along the latitudinal diversity
531 gradient. *Ecology Letters*, *21*(8), 1229-1236.
532 Gardner, T. A., Barlow, J., Araujo, I. S., Ávila-Pires, T. C., Bonaldo, A. B., Costa, J. E., ... & Peres, C.
533 A. (2008). The cost-effectiveness of biodiversity surveys in tropical forests. *Ecology*
534 *letters*, *11*(2), 139-150.
535 Gillett C, Johnson A, Barr I, Hulcr J. 2016. Metagenomic sequencing of dung beetle intestinal
536 contents directly detects and identifies mammalian fauna. bioRxiv DOI 10.1101/074849.
537 Gogarten, J. F., Hoffmann, C., Arandjelovic, M., Sachse, A., Merkel, K., Dieguez, P., ... &
538 Calvignac-Spencer, S. (2020). Fly-derived DNA and camera traps are complementary tools
539 for assessing mammalian biodiversity. *Environmental DNA*, 2(1), 63-76.
540 Gómez, A., & Kolokotronis, S. O. (2017). Genetic identification of mammalian meal source in
541 dung beetle gut contents. *Mitochondrial DNA Part A*, 28(4), 612-615.

Gotelli, N. J., & Colwell, R. K. (2001). Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. *Ecology letters*, *4*(4), 379-391.

Hughes, A. C., Orr, M. C., Ma, K., Costello, M. J., Waller, J., Provoost, P., ... & Qiao, H. (2021). Sampling biases shape our view of the natural world. *Ecography*, 44(9), 1259-1269.

ICMBio. (2019) Plano de Manejo Floresta Nacional do Tapajós: Volume I - Diagnóstico. 2019. Available in: https://www.gov.br/icmbio/pt-br/assuntos/biodiversidade/unidade-de-conservacao/unidades-de-biomas/amazonia/lista-de-ucs/flona-do-tapajos. Accessed in February 2024.

Keeping, D., & Pelletier, R. (2014). Animal density and track counts: understanding the nature of observations based on animal movements. *PLoS one*, 9(5), e96598.

Kelly, R. P., Port, J. A., Yamahara, K. M., Martone, R. G., Lowell, N., Thomsen, P. F., ... & Crowder, L. B. (2014). Harnessing DNA to improve environmental management. Science, 344(6191), 1455-1456.

Kerley, G. I., Landman, M., Ficetola, G. F., Boyer, F., Bonin, A., Rioux, D., ... & Coissac, E. (2018). Diet shifts by adult flightless dung beetles *Circellium bacchus*, revealed using DNA metabarcoding, reflect complex life histories. Oecologia, 188(1), 107-115.

Kiffner, C., Thomas, S., Speaker, T., O'Connor, V., Schwarz, P., Kioko, J., & Kissui, B. (2020). Community-based wildlife management area supports similar mammal species richness and densities compared to a national park. *Ecology and evolution*, 10(1), 480-492.

Kinoshita, G., Yonezawa, S., Murakami, S., & Isagi, Y. (2019). Environmental DNA collected from snow tracks is useful for identification of mammalian species. *Zoological Science*, 36(3), 198-207.

Kirtane, A. A., Wilder, M. L., & Green, H. C. (2019). Development and validation of rapid environmental DNA (eDNA) detection methods for bog turtle (*Glyptemys muhlenbergii*). *PloS one*, 14(11), e0222883.

Kocher, A., de Thoisy, B., Catzeflis, F., Huguin, M., Valière, S., Zinger, L., ... & Murienne, J. (2017). Evaluation of short mitochondrial metabarcodes for the identification of Amazonian mammals. *Methods in Ecology and Evolution*, *8*(10), 1276-1283.

570 Leempoel, K., Hebert, T., & Hadly, E. A. (2020). A comparison of eDNA to camera trapping for
571 assessment of terrestrial mammal diversity. *Proceedings of the Royal Society B*, 287(1918),
572 20192353.
573 Lenth R (2023). emmeans: Estimated Marginal Means, aka Least-Squares Means_. R package
574 version 1.8.5. https://CRAN.R-project.org/package=emmeans.
575 Lynggaard, C., Nielsen, M., Santos-Bay, L., Gastauer, M., Oliveira, G., & Bohmann, K. (2019).
576 Vertebrate diversity revealed by metabarcoding of bulk arthropod samples from tropical
577 forests. *Environmental DNA*, 1(4), 329-341.
578 Marques, T. A., Thomas, L., Martin, S. W., Mellinger, D. K., Ward, J. A., Moretti, D. J., ... & Tyack,
579 P. L. (2013). Estimating animal population density using passive acoustics. *Biological*
580 *Reviews*, *88*(2), 287-309.
581 Marsh, C. J., Louzada, J., Beiroz, W., & Ewers, R. M. (2013). Optimising bait for pitfall trapping of
582 Amazonian dung beetles (Coleoptera: Scarabaeinae). PLoS One, 8(8), e73147.
583 Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing
584 reads. *EMBnet. journal*, *17*(1), 10-12.
585 Masonick, P., Hernandez, M., & Weirauch, C. (2019). No guts, no glory: Gut content
586 metabarcoding unveils the diet of a flower-associated coastal sage scrub predator.
587 *Ecosphere*, 10(5), e02712.
588 Massey, A. L., Bronzoni, R. V. D. M., da Silva, D. J. F., Allen, J. M., de Lázari, P. R., dos Santos-
589 Filho, M., ... & Levi, T. (2022). Invertebrates for vertebrate biodiversity monitoring:
590 comparisons using three insect taxa as iDNA samplers. *Molecular Ecology Resources*,
591 22(3), 962-977.
592 Medina, A.M., Lopes, P.P. (2014). Resource utilization and temporal segregation of Scarabaeinae
593 (Coleoptera, Scarabaeidae) community in a Caatinga fragment. Neotropical Entomology.
594 43, 127-133.
595 McKee, A. M., Calhoun, D. L., Barichivich, W. J., Spear, S. F., Goldberg, C. S., & Glenn, T. C.
596 (2015). Assessment of environmental DNA for detecting presence of imperiled aquatic
597 amphibian species in isolated wetlands. *Journal of Fish and Wildlife Management*, 6(2),
598 498-510. https://doi.org/10.3996/042014-JFWM-034

599 Mora-Aguilar, E. F., Arriaga-Jimenez, A., Correa, C. M., da Silva, P. G., Korasaki, V., López-Bedoya,
600 P. A., ... & Noriega, J. A. (2023). Toward a standardized methodology for sampling dung
601 beetles (Coleoptera: Scarabaeinae) in the Neotropics: A critical review. *Frontiers in Ecology*
602 *and Evolution*, 11.
603 Nichols, J. D., & Karanth, K. U. (2011). *Camera traps in animal ecology: methods and*
604 *analyses* (Vol. 271). A. F. O'Connell (Ed.). New York: Springer.
605 Nichols, E. S., & Gardner, T. A. (2011). Dung beetles as a candidate study taxon in applied
606 biodiversity conservation research. *Ecology and evolution of dung beetles*, 267-291.
607 Nimalrathna, T. S., Fan, H., Quan, R. C., & Nakamura, A. (2023). Enhancing the dung beetle iDNA
608 tool for mammalian biodiversity monitoring and ecological studies. *Integrative*
609 *Conservation*, 2(3), 133-139.
610 Olds, B. P., Jerde, C. L., Renshaw, M. A., Li, Y., Evans, N. T., Turner, C. R., ... & Lamberti, G. A.
611 (2016). Estimating species richness using environmental DNA. *Ecology and*
612 *evolution*, 6(12), 4214-4226.
613 Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'hara, R. B., ... & Oksanen,
614 M. J. (2013). Package 'vegan'. *Community ecology package, version*, *2*(9), 1-295.
615 Pikitch, E. K. (2018). A tool for finding rare marine species. *Science*, *360*(6394), 1180-1182.
616 R Development Core Team. (2022) R: a language and environment for statistical computing. *R*
617 *Foundation for Statistical Computing*, Vienna.
618 Raine, E. H., Mikich, S. B., Lewis, O. T., Riordan, P., Vaz-de-Mello, F. Z., & Slade, E. M. (2018).
619 Extinctions of interactions: quantifying a dung beetle–mammal network. Ecosphere, 9
620 (11), e02491.
621 Ravetta, A. L., Brocardo, C. B. (2022) Primatas da Floresta Nacional do Tapajós e da Reserva
622 Extrativista Tapajós-Arapiuns. In: Brocardo, C. R., Giacomin, L. L. (Org). *Biodiversidade na*
623 *Floresta Nacional do Tapajós e na Reserva Extrativista Tapajós – Arapiuns*. Santarém, Pará:
624 UFOPA, 2022.
625 Riaz, T., Shehzad, W., Viari, A., Pompanon, F., Taberlet, P., & Coissac, E. (2011). ecoPrimers:
626 inference of new DNA barcode markers from whole genome sequence analysis. *Nucleic*
627 *acids research*, 39(21), e145-e145.

628 Ripple, W. J., Estes, J. A., Beschta, R. L., Wilmers, C. C., Ritchie, E. G., Hebblewhite, M., ... &
629 Wirsing, A. J. (2014). Status and ecological effects of the world's largest carnivores.
630 *Science*, 343(6167), 1241484.
631 Rodgers, T. W., Xu, C. C., Giacalone, J., Kapheim, K. M., Saltonstall, K., Vargas, M., ... & Jansen, P.
632 A. (2017). Carrion fly-derived DNA metabarcoding is an effective tool for mammal surveys:
633 Evidence from a known tropical mammal community. *Molecular Ecology Resources*, *17*(6),
634 e133-e145.
635 Salomão, R. P., Maia, A. C. D., Bezerra, B. M., & Iannuzzi, L. (2018). Attractiveness of different
636 food resources to dung beetles (Coleoptera: Scarabaeidae) of a dry tropical
637 area. *Neotropical entomology*, *47*, 69-78.
638 Saranholi, B. H., Rodriguez-Castro, K. G., Carvalho, C. S., Chahad-Ehlers, S., Gestich, C. C.,
639 Andrade, S. C., Freitas, P. D, & Galetti Jr, P. M. (2023). Comparing iDNA from mosquitoes
640 and flies to survey mammals in a semi-controlled Neotropical area. *Molecular Ecology*
641 *Resources*. https://doi.org/10.1111/1755-0998.13851.
642 Schnell, I. B., Bohmann, K., & Gilbert, M. T. P. (2015). Tag jumps illuminated–reducing sequence-
643 to-sample misidentifications in metabarcoding studies. *Molecular ecology resources*,
644 15(6), 1289-1303.
645 Scholtz, C. H., Davis, A. L. V., & Kryger, U. (2009). Evolutionary biology and conservation of dung
646 beetles (pp. 1-567). Sofia-Moscow: Pensoft.
647 Smith, J. A., Suraci, J. P., Hunter, J. S., Gaynor, K. M., Keller, C. B., Palmer, M. S., ... & Beaudrot, L.
648 (2020). Zooming in on mechanistic predator–prey ecology: Integrating camera traps with
649 experimental methods to reveal the drivers of ecological interactions. *Journal of Animal*
650 *Ecology*, *89*(9), 1997-2012.
651 Taylor, P. G. (1996). Reproducibility of ancient DNA sequences from extinct Pleistocene
652 fauna. *Molecular biology and evolution*, *13*(1), 283-285.
653 Tonelli, M. (2021). Some considerations on the terminology applied to dung beetle functional
654 groups. *Ecological Entomology*, 46(4), 772-776.

655 Varman, K. S., & Sukumar, R. (1995). The line transect method for estimating densities of large
656 mammals in a tropical deciduous forest: An evaluation of models and field
657 experiments. *Journal of Biosciences*, 20(2), 273-287.
658 Zhang, J. Kobert, K. Flouri, T. Stamatakis, A. (2014). PEAR: A fast and accurate Illumina Paired-
659 End reAd mergeR. *Bioinformatics,* 30(5): 614-620.
660 https://doi.org/10.1093/bioinformatics/btt593.

FIGURE CAPTIONS

Fig. 1. Study area and the dung beetle sampling scheme composed of the six transects with different killing solutions. a) Location of the study area, “Floresta Nacional de Tapajós”. b) Scheme of the six transects used for dung beetle collection (live trap: none killing solution; W+S: water salt; Etyl: ethylene glycol; alcohol: ethanol solution). c) Homemade pitfall trap used to capture dung beetles.

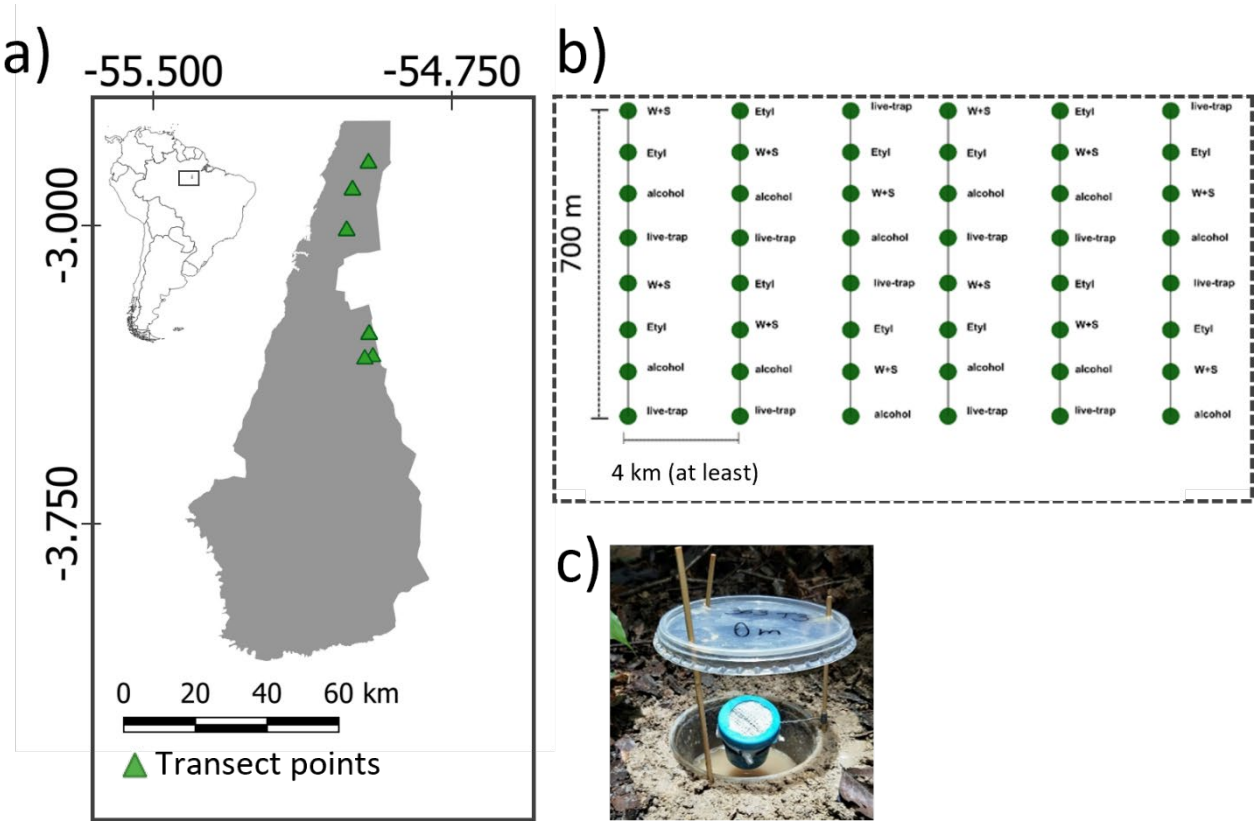
Fig. 2. Mammal species accumulation curves for 12SrRNA (red), 16SrRNA (blue), and both mini-barcodes (gray) against the number of dung beetle individuals. Error bars indicate standard errors of estimates.

Fig. 3. Results from a post-hoc analysis of a Poisson generalized linear mixed model. Lines represent comparisons of levels of ecological: beetle feeding strategy (A), beetle size (B), beetle nesting strategy (C); and methodological aspects: mini-barcodes (D), killing solution (E). Lines of levels that do not overlap are significantly different from one another at the 95% level. Significantly more mammals OTUs are found per specimen using the 16SrRNA mini-barcode compared to the 12SrRNA mini-barcode (D).

Fig. 4. Results obtained from post-hoc analysis of a negative binomial generalized linear model using emmeans. Lines represent comparisons of levels of mini-barcodes (A) and killing solutions (B), and levels whose lines do not overlap are deemed significantly different from one another at the 95% level. Significantly more mammal reads were retrieved using 16SrRNA than 12SrRNA (A), as well as using water or ethylene killing solution compared to ethanol.

685 **Fig. 1.**

686

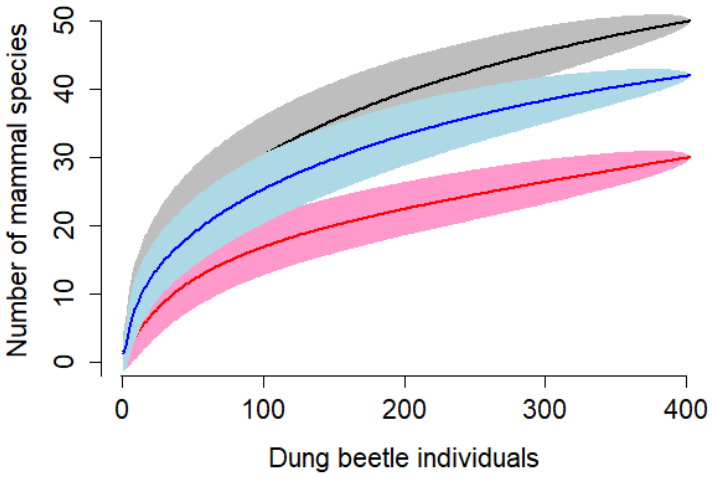


687

688

689 **Fig. 2.**

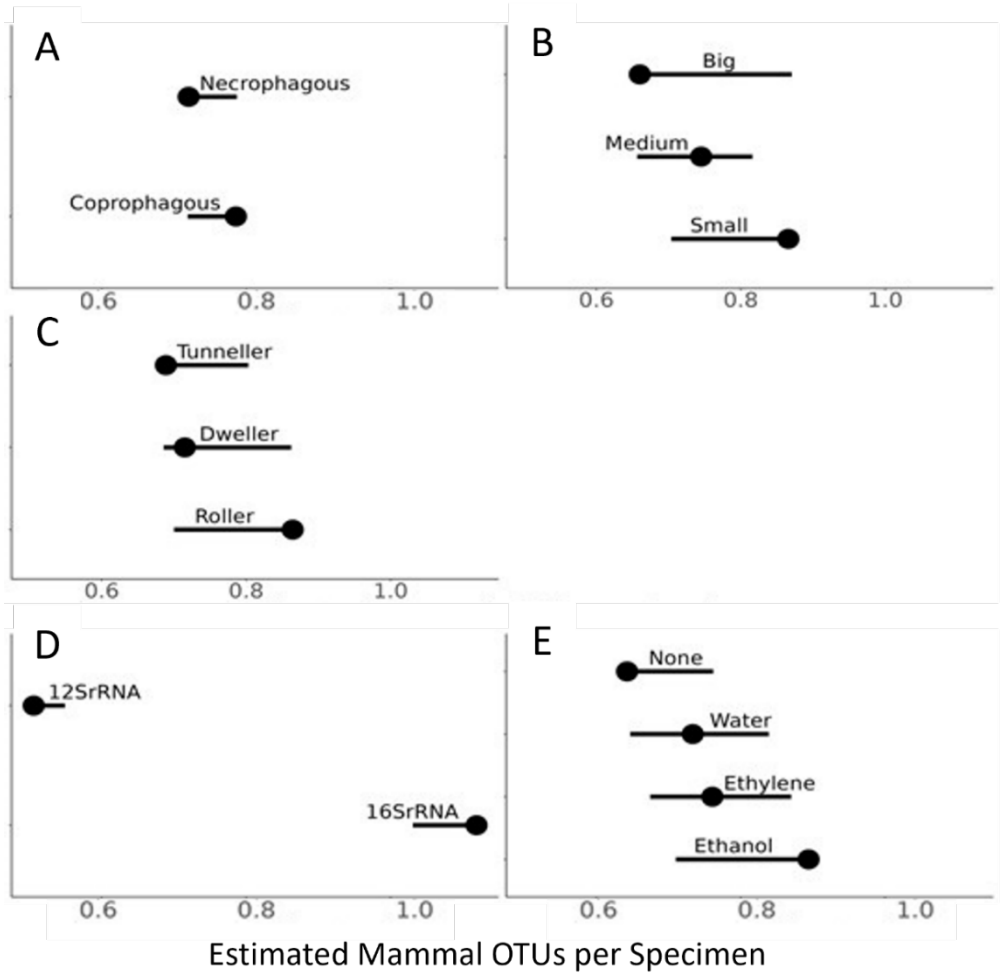
690



691

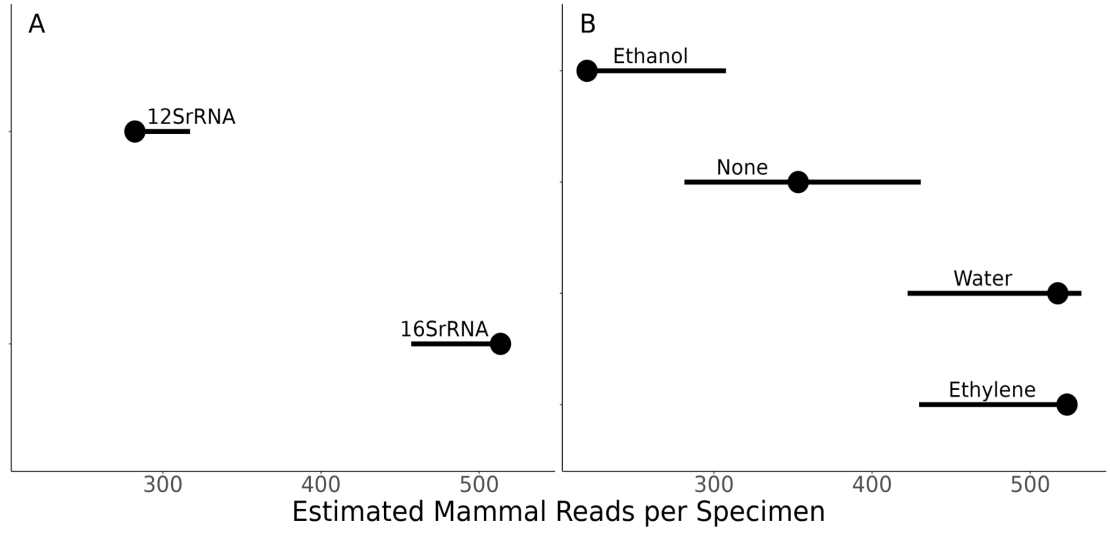
692 **Fig. 3**

693



694 **Fig.4**

695



696 **Table 1.** Mammal species detection and reads recovered from the iDNA from dung beetles using
697 12SrRNA and 16SrRNA mini-barcodes.

Taxon ID	Common name	Occurrence	Number of reads per OTUs (range per detection)		Detections by number of dung beetles			
			12SrRNA	16SrRNA	12SrRNA	16SrRNA	Total	Both mini-barcodes
Mammalia								
Artiodactyla								
Bos taurus	cattle	Domestic	16408 (22-3574)	41897 (27-9480)	40	55	76	19
Mazama sp.	brocket	Native	824 (31-171)	530 (74-456)	8	-	10	0
Pecari tajacu	collared peccary	Native	1830 (19-445)	1232 (53-350)	17	7	24	0
Carnivora								
Canis lupus	domestic dog	Domestic	6768 (18-2021)	11973 (16-3774)	26	34	46	14
Cerdocyon thous[1]	crab-eating fox	Native	81 (81-81)	21076 (17-2327)	1	73	73	1
Felis catus	domestic cat	Domestic	4440 (26-3314)	6123 (31-2456)	6	7	11	2
Herpailurus yagouaroundi[1]	jaguarundi	Native	200 (200-200)	122 (122-122)	1	1	2	0
Leopardus sp.	wild cat	Native	-	819 (819-819)	-	1	1	0
Nasua nasua	coati	Native	-	141 (29-112)	-	2	2	0
Panthera onca	jaguar	Native	1685 (36-434)	2224 (39-693)	9	14	23	0
Potos flavus	kinkajou	Native	-	199 (199-199)	-	1	1	0
Procyon cancrivoros[1]	raccoon	Native	-	15 (15-15)	-	1	1	1
Puma concolor	cougar	Native	228 (228-228)	5223 (16-2600)	1	14	15	0
Speothos venaticus	bush dog	Native	-	33 (33-33)	-	1	1	0
Chiroptera								
Pteronotus rubiginosus	mustached bat	Native	-	23 (23-23)	-	1	1	0
Cingulata								
Cabassous unicinctus[1]	southern naked-tailed armadillo	Native	19 (19-19)	-	1	-	1	0
Dasypus sp.		Native	42	9146	1	46	47	0

	long nosed armadillo		(42-42)	(18-953)				
Euphractus sexcinctus[1]	six-banded armadillo	Native	1325 (25-411)	221 (46-88)	9	3	11	1
Priodontes maximus	giant armadillo	Native	151 (28-53)	35 (35-35)	4	1	5	0
Didelphimorphia								
Didelphimorphia	marsupial	Native	1781 (1781-1781)	95 (95-95)	1	1	1	1
Didelphis sp.	opossum	Native	-	1407 (22-641)	-	5	5	0
Marmosops sp.	opossum	Native	842 (842-842)	-	1	-	1	0
Micoureus demerarae	woolly mouse opossum	Native	28 (28-28)	-	1	-	1	0
Lagomorpha								
Sylvilagus brasiliensis[1]	tapeti	Native	-	1529 (16-731)	-	8	8	0
Perissodactyla								
Tapirus terrestris	lowland tapir	Native	30 (30-30)	1044 (18-1026)	1	2	2	1
Pilosa								
Myrmecophaga tridactyla	giant anteater	Native	41 (41-41)	-	1	-	1	0
Tamandua tetradactyla	Southern tamandua	Native	-	102 (102-102)	-	1	1	0
Primates								
Primates	wild primates	Native	303 (37-124)	1078 (19-371)	4	7	10	1
Alouatta discolor[1]	red-handed howling monkey	Native	2193 (25-730)	7502 (16-2075)	11	23	27	7
Ateles marginatus[1]	white-cheeked spider monkey	Native	3615 (3615-3615)	2426 (57-2369)	1	2	2	1
Aotus azarai	Azara's night monkey	Native	2105 (42-665)	20852 (32-1939)	13	46	54	5
Mico argentatus[1]	silvery marmoset	Native	-	329 (54-133)	-	4	4	0
Plecturocebus sp.	titi monkey	Native	400 (400-400)	873 (27-530)	1	5	6	0
Saimiri ustus[1]	Golden-backed squirrel monkey	Native	1580 (31-1118)	3118 (42-1252)	5	9	12	2
Sapajus apella[1]	black-capped capuchin	Native	-	57 (57-57)	-	1	1	0
Rodentia								
Rodentia 1	wild rat	-	-	664 (164-315)	-	3	3	0
Rodentia 2	wild rat	-	-	233	-	2	2	0

				(33-200)				
Rodentia 3	wild rat	-	-	99 (44-55)	-	2	2	0
Rodentia 4	wild rat	-	-	2173 (152-1148)	-	3	3	0
Rodentia 5	wild rat	-	-	87 (87-87)	-	1	1	0
Rodentia 6	wild rat	-	-	354 (34-172)	-	3	3	0
Akodon sp.	wild rat	Native	-	64 (64-64)	-	1	1	0
Coendou prehensilis[1]	Brazilian porcupine	Native	40 (40-40)	19034 (15-2259)	1	54	55	0
Cuniculus paca	lowland paca	Native	166 (41-76)	-	3	-	3	0
Dasyprocta sp.	agouti	Native	299 (40-188)	-	3	-	3	0
Hydrochoerus hydrochaeris	capybara	Native	1759 (23-461)	9454 (19-1931)	15	32	43	4
Lonchothrix emiliae[1]	tuff-tailed spiny tree rat	Native	199 (199-199)	-	1	-	1	0
Oligoryzomys sp.	wild rat	Native	-	230 (101-129)	-	2	2	0
Colomys sp.	wild rat	Native	415 (32-325)	-	3	-	3	0
Trinomys sp.	spiny rat	Native	-	617 (20-597)	-	2	2	0
Total OTUs			30	42	-	-		
Total Reads			49797	174453	-	-		

[1]High match to more than one species or with a species not native to the region. Then, the species level was achieved after consulting the species with natural occurrence in the study site, based on the most recent inventories (Brocardo et al. 2022; Ravetta and Brocardo 2022), IUCN (International Union for Conservation of Nature) and GBIF (Global Biodiversity Information Facility).

SUPPORTING INFORMATION

Table S1. Detailed information about the 403 dung beetles analyzed.

Table S2. Pools of dung beetle iDNA for the metabarcoding sequencing.

Table S3. Non mammal species detection and reads recovered from the iDNA from dung beetles using 12SrRNA mini-barcode.

Fig. S1. Funnel pitfall trap preparation from 2-L plastic bottle.