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Invertebrates for vertebrate biodiversity monitoring: comparisons using three insect taxa as iDNA samplers

Running Head: Comparing iDNA sources for biodiversity monitoring

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Abstract

Metabarcoding of environmental DNA (eDNA) is now widely used to build diversity profiles from DNA that has been shed by species into the environment. There is substantial interest in the expansion of eDNA approaches for improved detection of terrestrial vertebrates using invertebrate-derived DNA (iDNA) in which hematophagous, sarcophagous, and coprophagous invertebrates sample vertebrate blood, carrion, or feces. Here, we use metabarcoding and multiple iDNA samplers (carrion flies, sandflies, and mosquitos) collected from 39 forested sites in the southern Amazon to profile gamma and alpha diversity. Our main objectives were to (1) compare diversity found with iDNA to camera trapping, which is the conventional method of vertebrate diversity surveillance and (2) compare each of the iDNA samplers to assess the effectiveness, efficiency, and potential biases associated with each sampler. In total, we collected and analyzed 1,759 carrion flies, 48,686 sandflies, and 4,776 mosquitos. Carrion flies revealed the greatest total vertebrate species richness at the landscape level, despite the least amount of sampling effort and the fewest number of individuals captured for metabarcoding, followed by sandflies. Camera traps had the highest median species richness at the site-level but showed strong bias towards carnivore and ungulate species and missed much of the diversity described by iDNA methods. Mosquitos showed a strong feeding preference for humans as did sandflies for armadillos, thus presenting potential utility to further study related to host-vector interactions.

Keywords: iDNA, carrion flies, sandflies, mosquitos, camera traps, biodiversity, metabarcoding

Introduction

Rapid and cost-effective biodiversity surveillance is a critical, burgeoning tool in ecology and conservation. DNA metabarcoding, or high-throughput DNA sequencing of taxonomically informative barcode genes, has become well-established to efficiently detect many species from bulk DNA samples for biodiversity surveillance. Such sequencing is now frequently used to catalog species from the DNA that organisms shed into the environment (eDNA) (Bohmann et al., 2014). For example, aquatic vertebrate or invertebrate communities can be detected with eDNA metabarcoding of water samples (Shaw et al., 2016; Stat et al., 2017; Valentini et al., 2016), animal diets can be

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detected from eDNA metabarcoding of feces (De Barba et al., 2014; Massey et al., 2021; Roffler et al., 2021), and plants and fungi can be detected by eDNA metabarcoding of pollen or ice core samples (Bell et al., 2019; Kraaijeveld et al., 2015; Varotto et al., 2021). Similarly, an emerging technique to monitor the diversity of terrestrial vertebrates uses animal-feeding invertebrates (whether feeding upon blood, flesh, or scat) as direct sources of species' DNA and is called invertebrate-derived DNA (iDNA) (Batovska et al., 2018; Calvignac-Spencer et al., 2013; Drinkwater et al., 2019, 2020; Gogarten et al., 2020; Kocher et al., 2017; Lee et al., 2016; Schnell et al., 2012; van der Heyde et al., 2020; Yu et al., 2012). Similar to camera traps, a conventional method for terrestrial vertebrate surveillance, metabarcoding blood, carrion, or fecal meals from iDNA samplers can be utilized as a non-invasive and efficient method at profiling a diversity of species in often remote or dense habitats. However, these methods differ in that camera trapping is strongly biased for the detection of medium to large terrestrial mammals, with lower detection probabilities of arboreal species, smaller bodied species, and other non-mammal taxa (Burton et al., 2015; O'Brien & Kinnaird, 2011). These drawbacks could make iDNA metabarcoding a more attractive method for biodiversity monitoring, particularly if iDNA is better able to detect the birds, amphibians, reptiles, small mammals, and arboreal mammals that camera trapping often fails to detect.

Leeches (Drinkwater et al., 2019; Schnell et al., 2012, 2018; Weiskopf et al., 2018) and carrion flies (Calvignac-Spencer et al., 2013; Lee et al., 2016; Lynggaard et al., 2019; Rodgers et al., 2017) have been the primary sources of validating iDNA metabarcoding for monitoring vertebrate biodiversity. The limited number of studies utilizing metabarcoding with leeches or carrion flies as iDNA samplers have shown that both taxa effectively profile vertebrate biodiversity in remote or difficult to access environments such as tropical forests (Drinkwater et al., 2020; Gogarten et al., 2020; Lee et al., 2016; Weiskopf et al., 2018). There is a growing literature comparing carrion flies as iDNA sources for metabarcoding to camera trapping (Gogarten et al., 2020; Lee et al., 2016; Rodgers et al., 2017) but even with their utility and effectiveness for profiling animal diversity, neither leeches nor carrions flies are widely collected for other research purposes (but see Hoffmann et al., 2016).

In contrast, some hematophagous insects such as mosquitos and sandflies are important pathogen vectors worldwide and are routinely sampled for disease surveillance, however they have been utilized less for profiling vertebrate diversity. Should these pathogen vectors be effective

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samplers of vertebrate biodiversity, then using iDNA to profile biodiversity would be an attractive supplemental and/or complementary pursuit to other study objectives using vector species (Batovska et al., 2018; Kocher et al., 2017). We are aware of only one study that has compared mosquitos and sandflies as iDNA samplers and found that both were seemingly effective at describing vertebrate diversity (Kocher et al., 2017). However, these results were not compared to data from a more established iDNA sampler or to more conventional methods of diversity surveillance.

A direct comparison of the emerging iDNA samplers (vectors such as mosquitos and sandflies) to the commonly utilized iDNA taxa groups (such as carrion flies or leeches) and finally to traditional methods of biomonitoring (such as camera traps) is needed to evaluate the diversity revealed by each iDNA source. Such a comparison will set the groundwork for assessing common biases, benefits, and shortfalls associated with each method given the current lack of comparison of established methods to the emerging iDNA samplers of mosquitos and sandflies. Invertebrate taxa differ in their feeding ecologies and other life history traits, which can introduce taxonomic biases into biodiversity assessments when these taxa are used as iDNA sources particularly when utilizing these methods with small sample sizes or spatial scales. Thus, this comparison is particularly needed at a spatial scale where these methods can be evaluated for both alpha and gamma diversity given the same suite of available species.

Here, we aim to assess the effectiveness of iDNA metabarcoding for biodiversity surveillance using landscape scale, bulk sampling of insect groups including carrion flies, sandflies, and mosquitos. We first compare the three iDNA samplers to camera trapping (the established method of vertebrate diversity surveillance) to determine if the two methods reveal similar species richness, composition, and relative abundance across the sampled landscape. Second, we compare the biodiversity found with each of the iDNA samplers to assess the efficiencies and potential biases with using different sources of iDNA as biomonitoring tools. To achieve these comparisons, we examine both gamma and alpha diversity with each iDNA sampler and tease apart the likely causes of dissimilarity.

Materials and Methods

Study area

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This study region near Sinop, Mato Grosso, Brazil (-12°04′73.9″S -55°43′85.0″W) represents topography and vegetation typical of the ecotone between the Cerrado (dominated by shrubland and grassland) and Amazonia (tropical forests) biomes (Mittermeier et al., 2003). The southern border of the Amazon rainforest was once contiguous dry, tropical forest that has been rapidly deforested by agricultural activity and urbanization. This has resulted in a checkerboard-like pattern of forest and agricultural patches (Fig. 1) with a mix of other matrix types including urban areas and secondary vegetation. Despite the rapid and widespread deforestation, this region hosts a great diversity of vertebrate species including tapir (*Tapirus terrestris*), capybara (*Hydrochoerus hydrochaeris*), giant anteater (*Myrmecophaga tridactyla*), crab-eating fox (*Cerdocyon thous*), harpy eagle (*Harpia harpyja*), maned wolf (*Chrysocyon brachyurus*), collared peccary (*Pecari tajacu*), jaguar (*Panthera onca*), brocket deer (*Mazama americana* and *Mazama gouazoubira*), and several monkey species (such as *Alouatta* spp., *Ateles* spp., and *Plecturocebus* spp.).

The area is largely flat and dominated by a hot and humid climate with daily temperatures showing greater fluctuation than annual mean low (23°C in June) and high (26°C in September) temperatures. The climate is dominated by dry (May to October) and wet (November to April) seasons with monthly precipitation averages ranging from a low of 2 mm in July to a high of 310 mm in February (climate data obtained to Sinop municipality).

Insect sampling

We collected bulk arthropods (sandflies, mosquitos, and carrion flies) in a coordinated effort at 39 fragmented forest sites from April – September in 2015 and 2016 (Fig. 1). At each site, we built a grid of three parallel 200 m transects with each transect approximately 50 m apart (see Appendix S1: Fig. S1 for trapping grid layout). Along each transect, we placed nine UV LED CDC light traps (BioQuip; Catalog Number: 2770) with approximately 30 m between traps for 27 total light traps per site. We also set three homemade carrion fly traps at each grid in a triangular arrangement with one trap at the end of the first transect, one at the beginning of the second transect, and the final trap at the end of the third transect (Appendix S1: Fig. S1). The number of traps differed between vectors and carrion flies because we predicted we would need to catch many more mosquitos and sandflies to ascertain the same level of vertebrate biodiversity and because mosquito and sandflies were also

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captured for a landscape epidemiology project. We engineered each carrion fly trap from two 2 L soda bottles and gauze mesh. The trap was designed to bait carrion flies with the smell of rotting meat (both beef and pork) while also keeping the trapped carrion flies physically separated from the bait (see Appendix S1: Fig. S2 for carrion fly trap design). We trapped and collected the insects over the course of 4 days and 3 trap nights at each site. We checked traps each day and collected the collection cups containing the live insects (and replaced this with a new collection cup) every 24 hours; we placed the collection cups containing the live insects in a portable refrigerator during transportation to Universidade Federal de Mato Grosso (UFMT, Sinop campus) lab facilities. We immediately transferred insect collections to a -20°C freezer for at least 30 minutes to stun the insects in order to then separate out sandflies and mosquitos from other insect by-catch. We sorted sandflies, mosquitos, and carrion flies into separate pools based on site and date into 2 mL microtubes. Based on managing the total number of DNA extractions as well as the manufacturer's suggestions for amount of material per extraction, we pooled sandflies at 50 individuals per sample, mosquitos at fifteen individuals per sample, and carrion flies at five individuals per sample. Finally, we placed sorted insects into a -80°C freezer until they were shipped using dry ice to our home lab facility at Oregon State University where they were once again frozen at -80°C until molecular processing.

DNA extraction, amplification, and sequencing

Prior to DNA extraction, we homogenized pooled samples in buffer solution using ceramic beads (sandflies) and disposable tissue grinding pestles (mosquitos). To macerate carrion flies, we used the blunt-end of a disposable tipped applicator to break apart each individual followed by immediately adding the buffer solution. We extracted DNA from sandflies and carrion flies with the Qiagen Blood and Tissue Kit (Qiagen, Hilden, Germany) with slight modifications to the manufacturer's specifications. Briefly, 200 uL of Buffer ATL and 20 uL of Proteinase K were added to the sample in a 1.7 mL Eppendorf tube and the sample incubated for 3-5 hours at 56°C. Post-incubation, samples were vortexed for 10 minutes and then purified through washing. The DNA was eluted in a final volume of 100 uL. Because we wanted to isolate both DNA and RNA from mosquitos for separate studies of viral surveillance, we extracted nucleic acids following a phenol-chloroform protocol modified from Griffiths et al. (2000) and Simister et al. (2011) with final ethanol

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precipitation and purification to isolate DNA. To purify for DNA, we resuspended the nucleic acid pellet in 50 uL of RNase-free water and incubated half the sample (25 uL) with RNase A in a final concentration of 100 ug/mL for 10 minutes at 37°C. The other half of the sample was purified for RNA and then synthesized for cDNA.

We amplified the extracted DNA from each iDNA sampler type in two separate reactions using a slight modification of the pan-vertebrate primer pair 12SV5F/12SV5R (Riaz et al., 2011), which targets approximately 100 bp in the 12S region of the vertebrate mitochondrial genome. We used the reverse primer 12SV5R (TTAGATACCCCACTATGC) as Riaz et al. (2011) and a modified version (we change the thymine to a degenerate base shown underlined) of the forward primer 12SV5F to allow for broader binding of vertebrate targets (YAGAACAGGCTCCTCTAG). The primers were dual-indexed with identical 8 bp sequences to remove tag jumping errors and chimeras during sequence analysis and a 3 bp tail of Ns to increase sequence diversity and prevent digestion of index nucleotides during library preparation. Each PCR replicate within a library received a unique index, which allowed us to determine the consistency of taxonomic identification among replicates. In brief, PCR reactions were carried out in a volume of 20 uL using 10 uL AmpliTaq Gold 360 Master Mix (final concentration of 1x), 5 uL of forward and reverse primers (final concentration of 0.25 uM), 3 uL of water, and 2 uL of DNA template. PCR cycling was as follows: initial denaturing at 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds and 58°C for 30 seconds and 72°C for 30 seconds, and a final extension at 72°C for 7 minutes.

PCR amplicons were cleaned using PCRClean DX solid-phase reversible immobilization magnetic beads (Aline Biosciences, Woburn, MA, USA). Each PCR reaction was quantified using Accublue High Sensitivity dsDNA Quantitation kit (Biotium, Fremont, CA, USA) and normalized to 6 ng/uL. Each group of 384 PCR products was then pooled into a single library and individual libraries were then tagged with an additional 6 bp identifying index using the NEBnext Ultra II DNA Library Prep kit (New England Biolabs, Ipswich, MA, USA). Pooled samples were analyzed on a bioanalyzer to confirm fragment size. The libraries were then sequenced using the Illumina HiSeq 3000 (Illumina, San Diego, CA, USA) at the Center for Genome Research and Biocomputing at Oregon State University.

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Sequence analysis

We analyzed raw sequence reads using a bioinformatics pipeline designed to trim and sort the sequence reads according to scat sample identification. An outline of the bioinformatic process is as follows: (1) raw reads were paired using PEAR software (Zhang et al., 2014); (2) followed by demultiplexing using 8 bp index sequences unique to each sample (mismatches discarded) with a novel grep regular expression (see Massey et al., 2021); (3) lastly, unique OTUs from each sample were counted and taxonomically assigned to the best match in NCBI Genbank using BLAST (www.ncbi.nlm.nih.gov/blast) against 12S vertebrate sequences available in GenBank.

We carried out a series of filtering and quality control measures on taxonomically assigned sequences. For each of the three iDNA datasets, we first compared query sequences found in our samples with query sequences found in our negative controls. The majority of sequences in the negative controls corresponded to human DNA so we removed these contaminant reads and any other human DNA sequences that appeared in our samples. Additionally, we removed sample replicates that did not amplify (below a 500 read threshold). We then removed OTU's with either a percent identity score less than 90% or 1% of the total number of sequences in that sample. Finally, we eliminated species that were not found in both sample replicates. Because there is a lack of a comprehensive reference library for all vertebrate species in this highly biodiverse region, we manually reviewed BLAST results for each purported species to ensure that the 12S barcode discriminated species from sympatric congeners or confamilials and to confirm that the taxonomic assignments were for species regional to Mato Grosso, Brazil (IUCN and eBIRD databases were used to check species ranges). For species with high percent matches (98-100%), we retained species level assignments if there were no better or equal percent matches for other species. Species level assignments were changed to genus or family level assignments if we found an equal match for another regional congener or confamilial species. For percent matches between 90-97.99%, we changed all species level assignments to the genus or family level assignments. If no suitable matches were discovered at the species, genus, or family level, we removed these sequences from our datasets.

Camera traps

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To compare metabarcoding results to methods traditionally used for profiling animal abundance and/or occurrence, we used digital infrared camera traps (Bushnell®) to sample vertebrate diversity from May 2015 - March 2016 at 33 of the 39 sites where we collected arthropods as iDNA sources. In total, we deployed 116 camera traps; cameras per site varied from two to seven although at the majority of sites three cameras were utilized. We set up the camera traps 40 cm above the ground and they were active 24-hours per day for a total of 5,093 camera days. Camera days per camera ranged from 26 – 92 with the average number of camera days equal to 44 days.

Data analysis

Sample-size-based rarefaction curves with extrapolation (Chao et al., 2014) were created using the iNEXT (datatype = "incidence_raw") and ggiNEXT functions from the iNEXT package in R (Chao et al., 2014; Hsieh et al., 2016) to examine species richness across the three iDNA datasets and from the camera trapping data. For the iDNA data, we converted species read abundance to species incidence data (presence/absence) at the sample level. Due to differences in the number of amplified samples across the three iDNA sources, species richness was extrapolated to the iDNA source with the greatest number of samples following Chao et al. (2014). For the camera trapping data, we measured species richness across the total number of camera days from all deployed cameras.

We used a relative abundance index (RAI) to compare the biodiversity found using iDNA metabarcoding data and camera trap data. For metabarcoding data, the RAI is equal to the sum of occurrences for species *i* divided by the total number of pooled samples. The RAI measured from camera trapping data is calculated as the number of species *i* events divided by the total number of camera trap nights. A single species event from camera trap data was defined by any individual (or group of individuals) from one species captured by a camera over a one-hour time frame. We then used RAI to visualize the gamma diversity according to both iDNA and camera trapping methods by binning species by important life history traits to compare the effectiveness of the biomonitoring methods among taxonomic groups. To compare RAI across iDNA and camera traps, we only included sites that were sampled by both methods.

After comparing diversity from iDNA and camera traps, we then compared the iDNA sources to each other to assess the similarities, efficiencies, and potential biases of each sampler type.

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Utilizing the *vegan* package (Oksanen et al., 2013) in R, we used a Bray-Curtis dissimilarity matrix of sites x species and applied a PERMANOVA test using the *adonis* function to test for significance in the separation of the vertebrate community based on iDNA sampler. We then used the *metaMDS* function to visualize dissimilarities in species composition at sites using non-metric multidimensional scaling (NMDS). Each site was designated three rows in our abundance matrix: one for each iDNA type (carrion fly, sandfly, or mosquito). We also utilized the *ordiellipse* function to visualize the spread of the data based on iDNA source with ellipses drawn to two standard deviation (95% confidence intervals) from the group mean. Lastly, significant species vectors were calculated using the *envfit* function.

To answer whether an iDNA sampler could replace another iDNA sampler in profiling alpha diversity, we calculated RAI of species at the site level for each of the iDNA datasets and compared species occurrences at sites from the different iDNA samplers. We visualized the results in scatterplots. If two iDNA datasets showed similar species composition at sites, most data points would fall along an isometric line. If two iDNA samplers revealed dissimilar species composition across sites, more data points would fall along the x-axis or y-axis revealing occurrence of a species in one of the iDNA datasets and an absence of that species in the other iDNA dataset. Finally, to further explore the different feeding ecologies associated with the insect taxa used as iDNA samplers, we compared the RAI at sites for the most abundant species in each iDNA dataset using boxplots.

Human DNA

When analyzing metabarcoding data for profiling vertebrate diversity, we remove all human DNA reads under the assumption that they are contamination. However, a disproportionate proportion of the DNA sequences from mosquito pools was identified to be human, and the total human read abundance in samples was well above the level of human DNA found in the extraction and PCR negative controls. Thus, we reexamined levels of human DNA found in all three iDNA metabarcoding datasets. We first eliminated non-human contaminants and non-amplifying samples (replicates with less than 500 total reads). With the remaining dataset, we culled all non-human read sequences. We closely examined the amount of human DNA in the extraction and PCR negative controls to determine a read threshold for a human positive sample. To be extremely conservative in what we

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deemed as a sample positive for human, we set the threshold as the highest read count in any negative control replicate for each iDNA dataset. For example, if the highest read count for human DNA in a negative control for the carrion fly metabarcoding data was 25,000 then the threshold to be counted as a sample positive for human DNA was 25,000 reads in a replicate. Additionally, we also eliminated samples where human DNA was not present in both sample replicates. A relative abundance index (RAI) was calculated as the number of samples positive for human DNA at a site divided by the total number of samples at a site.

Results

Carrion fly 12S metabarcoding resulted in 16,130,638 paired sequence reads from 368 pooled samples. The average read depth per sample replicate was 21,976 reads. The sandfly 12S metabarcoding results showed 25,093,673 paired sequence reads from 943 pooled samples and the average read depth per sample replicate is 13,780 reads. Finally, mosquito 12S metabarcoding results resulted in 35,132,064 total paired sequence reads from 322 pooled samples with an average read depth per sample replicate of 55,152 reads.

After conservative quality measures designed to clean the raw data, the following datasets were used for analysis (and excluded human DNA): carrion fly data had 5,389,372 total paired sequences from 271 pooled samples which represented 37 of the 39 sites; the sandfly data had 5,436,898 total paired sequences from 354 pooled samples which represented 36 sites; finally, the mosquito data had 7,077,247 paired sequences from 82 pooled samples which represented 26 sites (a summary of site-level data for each iDNA method can be found in Appendix S1: Table S1). Pooled samples of carrion flies showed the highest amplification success followed by pooled sandfly samples and finally pooled mosquito samples (Table 1), although these results exclude human DNA which made up the majority of sequence reads in the mosquito dataset.

In total, iDNA and camera trapping methods revealed 99 vertebrate taxa across the sampled landscape (Table 2). iDNA alone described 89 taxa with carrion fly data describing the most diversity of any sampler with 66 taxa while sandfly data revealed 53 taxa and mosquito data revealed 20 taxa.

Camera traps generated 40,490 images from the 116 total camera traps of which 33,616 images contained vertebrate species that could be confidently identified to the species level. Camera

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trap data revealed 28 vertebrate species and added 10 species to the diversity already described with iDNA.

Comparing biodiversity found with insect samplers and camera traps

Species accumulation curves show a statistically significant difference in species richness gleaned from each iDNA source and camera trapping (Fig. 2A). Using carrion flies as a measure of biodiversity more rapidly accumulates vertebrate species across the landscape than both sandflies and mosquitos. Additionally, the total number of carrion flies analyzed (n = 1,759) was lower than both the total number of sandflies (n = 48,686) and mosquitos (n = 4,776) analyzed. The species accumulation curve for camera trap data shows that species richness plateaus after approximately 1,500 camera days meaning that few to no new species are revealed by camera traps even with continued camera days (Fig. 2A). The species richness from camera traps was most similar to the species richness from the extrapolated curve for the pooled mosquito samples, which was the least efficient iDNA sampler in describing vertebrate diversity (Fig. 2A; Table 1). Collating species incidence data for iDNA samplers at the site level showed a similar pattern (Fig. 2B). Carrion flies were the single best iDNA sampler for describing the greatest species richness, but the greater sampling effort for sandflies led to less divergence across sites than across samples (Fig. 2A, B). In contrast, despite the same sampling effort, the higher number of sandflies than mosquitos captured led to greater divergence in interpolated species richness by site. Thus, mosquito iDNA added little species richness beyond that detected by sandflies (Fig. 2B).

Comparisons of species' RAI from iDNA samplers and camera traps show differences in diversity profiles across taxa groups (Fig. 3). As expected, camera traps showed a high diversity of carnivore and ungulate species and a low diversity of arboreal species or birds. Camera traps also did not capture the occurrence or diversity of domestic species or rodents particularly when compared to the diversity of these groups found using iDNA methods. Although not supported by our extraction and PCR negative controls, we caution that domestic species are sometimes detected as a result of contamination. Carrion fly data consistently showed a high diversity of species in each taxa group. Sandfly data also showed high diversity of species in most taxa groups with an especially high diversity and relative abundance of armadillo species (Fig. 3; Table 2). Lastly, mosquito data showed

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the lowest species diversity for many of the taxa groups and only showed a high diversity of domestic species when comparing mosquito data to the other datasets (although carrion fly data still displayed greater diversity and relative abundance from domestic species). When species incidence data was parsed at the site level, camera traps showed the highest median species richness at sites (Fig. 4) and a consistent diversity profile across sites (Appendix S1: Fig. S3) indicating that camera trapping consistently samples the same species.

Comparing the different iDNA samplers for biomonitoring

A PERMANOVA test and further visualization of the dissimilarity matrix using NMDS (k = 2; stress = 0.17) show that the community composition did not differ significantly based on iDNA source (p-value = 0.25) (Fig. 5). Although there was no statistically significant separation in the community composition found using the different iDNA methods, a site-by-site comparison of the alpha diversity found with each iDNA method does not support substantial overlap of the vertebrate communities revealed by each iDNA source at the site-level (Fig. 5). Although carrion flies revealed the greatest gamma diversity, when comparing species presence at a site across iDNA datasets, we found that there were many species absences in the carrion fly data at sites where sandflies or mosquito samplers recorded a species presence (Appendix S1: Fig. S4). Carrion fly data missed 189 of the 221 species occurrences from the sandfly data and 49 of the 70 total species occurrences from the mosquito data when examined at the site-level (Appendix S1: Fig. S4). Arrowed vectors show that the significant species in the ordination (p-value < 0.001) were also the most abundant in each iDNA dataset, and thus these species likely drive the separation in the vertebrate communities at sites as described by the different iDNA samplers (Fig. 5). These significant, non-human vertebrate species include cattle (highest RAI in carrion fly data), dog (highest RAI in mosquito data), nine-banded armadillo (highest RAI in sandfly data), capuchin, lesser anteater, and a species from the Phasiandidae family (likely chicken; second highest RAI in mosquito data). This sampler bias was confirmed by our analysis of the most abundant species in each of the iDNA datasets, which revealed the differing feeding associations of each insect group (Fig. 6), and we found that these abundant species were responsible for many of the site-level mismatches between the iDNA samplers (Appendix S1: Fig. S4). Additionally, we determined that humans were the primary feeding target of

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mosquitos (Fig. 6) and human DNA accounted for more than 80% of the total sequence reads in the mosquito iDNA dataset.

Discussion

This study provides evidence that carrion flies are the superior method for landscape scale biodiversity surveillance compared to both camera traps and the other iDNA sampling methods (carrion fly iDNA identified 66 vertebrate species, sandfly iDNA detected 53 species, mosquito iDNA detected 20 species, and camera traps identified 28 species). Previous work has found that iDNA from carrion flies produced complementary results when compared to more traditional methods of biomonitoring such as camera traps (Gogarten et al., 2020; Rodgers et al., 2017). We found a similar pattern to Rodgers et al. (2017) in their comparison of carrion flies to existing camera trap data in that even with a small sample size and effort, carrion flies used as iDNA samplers were able to profile most of the diversity that had been found with long-term camera trapping while also revealing new species diversity. However, with our larger sampling effort, we were able to show that carrion flies were much more effective at describing diversity at the landscape scale and species richness likely would have increased for each iDNA sampler if we had increased our sampling effort, particularly at the site level, whereas species richness had fully saturated by 1,500 camera-days (Fig. 2 and Fig. 4).

While camera traps revealed lower gamma diversity than carrion flies and sandflies, they had the greatest median species richness at sites (alpha diversity) compared to each of the iDNA samplers. This points to camera traps reliably revealing the same suite of species from across sites resulting in higher alpha diversity but lower overall gamma diversity when compared to iDNA samplers. The missing species richness exhibited by camera traps at the landscape scale of our study is likely due to camera traps preferentially targeting larger-bodied mammals (Burton et al., 2015; O'Brien & Kinnaird, 2011). We found support for this as camera traps revealed the greatest diversity of carnivore species and ungulate species while missing most of the iDNA-described diversity of the arboreal species and the smaller-bodied mammals.

The improved effectiveness (biodiversity detection) with carrion flies over other iDNA sources and camera traps comes with additional advantages in efficiency (time and labor). Carrion flies can be trapped with inexpensive, homemade traps compared to the more expensive camera traps

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or the UV LED CDC light traps used to capture sandflies and mosquitoes. These homemade traps also did not require batteries compared to the UV LED CDC light traps which require 6V batteries that need to be changed approximately every 24 hours. Our trap design (Appendix S1: Fig. S2) was effective at attracting and trapping carrion flies while at the same time preventing individuals from accessing the bait. Using a blocking primer for the species used as bait is also an option when utilizing carrion flies as an iDNA source. Carrion flies are thus much more amenable to sampling in remote landscapes where transporting and shipping pre-made traps or bulky equipment can be difficult. Carrion flies were also the easiest iDNA sampler to sort. We rarely caught bycatch in the carrion traps and were able to easily group individuals into pooled samples. Sorting sandflies and mosquitos was more laborious to separate these taxa from other invertebrate bycatch. Finally, we identified the most species with carrion flies compared to the other iDNA samplers despite a much lower sampling effort of 3 traps per site compared to 27 traps per site for mosquitos and sandflies, which resulted in much fewer individual carrion flies (n = 1,759) compared to the total number of sandflies (n = 56,774) and mosquitos (n = 4,776). Carrion fly samples had the greatest amplification efficiency of non-human vertebrates (Table 1) despite only 5 individual flies coextracted compared with 15 mosquitos and 50 sandflies, although amplification efficiency reflected all captured sandflies and mosquitos (not only blood-fed females) and did not include human DNA, which was highest in mosquito samples.

Although carrion flies were the most effective iDNA sampler, we wanted to examine whether the gamma and alpha diversity described varied substantially among iDNA sources to assess the effectiveness of sandflies and mosquitos as biodiversity samplers. We found that the overlap in vertebrate communities from each iDNA source was generally supported at the landscape scale, however, the different iDNA sources detected different communities of species at each site, and the same sites were typically distant in ordination space. Although carrion flies were the most effective sampler for detecting gamma diversity, we found carrion flies were less redundant with mosquitos and sandflies for detecting local biodiversity and carrion flies missed 85% and 70% of the species occurrences from across all sites in the sandfly data and in the mosquito data respectively, although most of these misses corresponded to the dominant species in either the sandfly or mosquito data as

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shown in Appendix S1: Fig. S4. This points to needing a substantially larger sampling effort at the site level for iDNA methods, particularly carrion flies, to saturate species detections.

Aside from the effect of sampling effort on diverging alpha diversity profiles, some of the mismatch is explained by the differing feeding ecologies of each insect group. We detected substantial taxonomic biases in vertebrate detections among iDNA sources that were likely driven by differences in the host preferences of the common mosquito and sandfly species. Aside from domestic dog, we found evidence of contrasting feeding preferences for vector taxa that were consistent with their known relationships with host species. Sandflies overwhelmingly targeted armadillos (*Dasypus novemcinctus* and *Dasypus kappleri*) which aligns with our understanding of both the relationship between armadillos and sandflies (Akhoundi et al., 2016; Alexander, 2000; Lainson et al., 1979) as well as armadillos as an important host species for the life cycle of *Leishmania* parasites (Lainson et al., 1979; Lainson & Shaw, 1989), which are transmitted by female sandfly vectors. Kocher et al. (2017) reported the first direct evidence of sandfly-armadillo interaction using metabarcoding of sandflies, and our findings overwhelmingly support this finding. Mosquitos also exhibited strong host preferences for humans (likely members of our field teams working within the forest patches), which even after rigorous quality control to eliminate contamination was the dominant iDNA data source representing 80% of the total reads. While many species of mosquito are opportunistic feeders, there are a number of species that target humans including *Aedes aegypti* which is the main vector for dengue, yellow fever, and chikungunya and is found throughout tropical and sub-tropical regions worldwide (McBride, 2016; Zwiebel & Takken, 2004). Interestingly, our metabarcoding data is corroborated by previous studies spanning the Amazon region that show upwards of 80% of *A. aegypti* bloodmeals contain human DNA (McBride, 2016). Compared to the targeted feeding behavior of sandflies and mosquitos (as well as leeches shown by Drinkwater et al. 2019), the feeding ecology of carrion flies may offer the least biased view of biodiversity across a landscape. This is because their feeding behavior is likely driven by the presence of carrion and/or scat rather than an attraction to particular species (Calvignac-Spencer et al., 2013; Rodgers et al., 2017). Given these findings, the feeding ecologies of each insect group should be an important consideration because choice of iDNA sampler can lead to mismatches in the vertebrate diversity profiles, especially with smaller sampling sizes.

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By exploring the biases associated with each sampling type, we found evidence that carrion flies are significantly more effective in describing landscape level biodiversity when compared to diversity found with camera traps and other iDNA samplers, even with the least sampling effort and smallest sample size. We found that camera traps predictably were biased towards larger-bodied mammals and away from arboreal and smaller-bodied species, thus missing much of the biodiversity described with iDNA samplers, but also had the highest species richness at the site-level. Sandflies and mosquitos show feeding behavior targeted at armadillos and humans, respectively. Although sandflies and mosquitos were inferior samplers for large-scale biodiversity surveillance compared to carrion flies, they can add value to existing host-vector-pathogen surveillance efforts. The general feeding preferences shown by the vector taxa support the known ecological relationships between these invertebrate taxa and their hosts, which is particularly relevant in tropical forest frontiers where vectors maintain enzootic sylvatic cycles, which can cause disease spillover into humans and/or domestic animals (Figueiredo, 2007; Vasconcelos et al., 2001). With advances in high-throughput sequencing, the steadily decreasing expense of metabarcoding, and most importantly improvement of taxonomic reference databases for DNA barcoding, iDNA can be an effective and efficient method for biomonitoring.

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Data Accessibility

All metabarcoding data collated for analyses will be available as spreadsheets in the Supplemental Information.

Author Contributions

ALM led project design, fieldwork, laboratory work, analysis, and writing. RB was involved with project conceptualization and fieldwork and laboratory work design. DJFS was instrumental in assisting with fieldwork and data collection. JA helped with early laboratory protocols and library preparation for metabarcoding. PRL and CSSB carried out camera trapping and image processing and provided raw camera trap data. MSF and GRC helped with project conceptualization and design. CP and TL were involved with project conceptualization and project design and provided substantial feedback on the manuscript.

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Table 1: Efficiency of each insect taxa as an iDNA sampler used to profile vertebrate diversity from the blood (sandflies and mosquitos) or carrion meals (carrion flies). Human DNA is excluded, which lowers the total number of amplified mosquito samples and consequently the percentage of usable samples.

	carrion flies	sandflies	mosquitos
Total number of traps	117	1053	1053
Total number of individuals captured (and processed)	1,759 (1,759)	56,774 (48,686)	4,776 (4,776)
Total number of pooled samples processed for metabarcoding	370	976	321
Total number of amplified samples used for analysis post quality control measures	271	354	82
Percentage of usable samples	73.2%	36.3%	25.5%

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Table 2: Vertebrate diversity found using iDNA samplers and camera trapping methods. Values show a species' RAI in a given dataset. An "X" indicates that a species was present at a RAI value less than 1% (a rare species).

		iDNA: carrion fly	iDNA: sandfly	iDNA: mosquito	Camera traps
Mammals					
Artiodactyla					
Bos taurus	cattle	**0.41**	**0.02**	**0.03**	
Mazama americana	red brocket	**0.01**	**0.01**		**X**
Mazama gouazoubira	brown brocket				**0.03**
Mazama nemorivaga	Amazonian brown brocket	**0.01**		**X**	
Mazama spp.	deer				**0.01**
Ovis aries	sheep	**0.03**			
Pecari tajacu	collared peccary	**0.04**	**0.01**	**0.01**	**0.03**
Tayassu pecari	white-lipped peccary	**0.04**	**0.01**		**0.01**
Carnivora					
Canidae (family)		**0.02**	**X**		
Canis lupus familiaris	domestic dog	**0.27**	**0.18**	**0.17**	**X**
Cerdocyon thous	crab-eating fox				**0.01**
Eira barbara	tayra	**0.01**			**X**
Felidae (family)	wild cats		**0.02**		
Felis catus	domestic cat	**0.01**	**0.01**	**0.01**	**X**
Leopardus	ocelot				**0.01**

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pardalis					
Nasua nasua	coati	**0.03**	**X**	**X**	**0.01**
Panthera onca	jaguar				**X**
Puma concolor	cougar		**0.01**		**X**
Puma yagouaroundi	jaguarundi				**X**
Chiroptera					
Micronycteris spp.	big-eared bats		**X**		
Phyllostomus hastatus	greater spear-nosed bat	**X**	**X**		
Rhogeessa spp.	vesper bats			**X**	
Vespertilionidae (family)	vesper bats		**X**		
Dasypodidae					
Cabassous unicinctus	southern naked-tailed armadillo	**0.01**			**X**
Dasypus kappleri	greater long-nosed armadillo		**0.05**		
Dasypus novemcinctus	nine-banded armadillo	**0.01**	**0.22**	**0.01**	**0.04**
Dasypus septemcinctus	seven-banded armadillo		**0.01**		
Dasypus spp.	long-nosed armadillos		**0.02**	**X**	
Euphractus sexcinctus	six-banded armadillo	**0.01**			**X**
Myrmecophaga tridactyla	giant anteater		**X**	**X**	**X**

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Priodontes maximus	giant armadillo				**X**
Didelphimorphia					
Caluromys lanatus	brown-eared woolly opossum	**0.04**	**X**		
Caluromys philander	bare-tailed woolly opossum	**X**			
Didelphidae (family)	American opossums	**X**	**X**		
Didelphis spp.	large American opossums		**0.01**		
Gracilinanus spp.	opossum	**0.01**			
Marmosa murina	common mouse opossum	**0.01**			
Metachirus nudicaudatus	brown four-eyed opossum	**0.02**	**X**		
Micoureus demerarae	woolly mouse opossum		**X**		
Philander spp.	gray and black four-eyed opossums		**X**		
Lagomorpha					
Leporidae (family)	hares, rabbits		**0.01**		
Perissodactyla					
Tapirus terrestris	Brazilian tapir	**0.10**	**0.04**	**0.01**	**0.09**
Pilosa					
Tamandua tetradactyla	southern anteater	**X**	**0.02**		**X**

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Primates					
Ateles belzebeth	white-bellied spider monkey	**0.03**	**0.01**		
Callicebus spp.	titi monkeys	**X**			
Callitrichidae (family)	marmosets, tamarins	**0.02**			
Cebidae (family)	new world monkeys	**X**			
Cebus spp.	capuchin	**0.12**	**0.01**	**0.01**	**0.01**
Chiropotes albinasus	white-nosed saki		**X**		
Plecturocebus vieirai	Vieira's titi				**X**
Rodentia					
Coendou prehensilis	Brazilian porcupine	**0.01**			**X**
Coendou spp.	hairy dwarf porcupines		**0.01**		
Cricetidae (family)	new world rodents	**0.01**	**X**		
Cuniculus paca	lowland paca	**X**	**0.01**		**0.04**
Dactylomys dactylinus	Amazon bamboo rat	**X**			
Dasyprocta azarae	Azara's agouti				**0.06**
Dasyprocta spp.	agoutis	**0.01**		**0.01**	
Hydrochoerus hydrochaeris	capybara	**0.03**	**0.01**		**X**
Makalata	Brazilian spiny	**X**			

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didelphoides	tree-rat					
Mus musculus	house mouse	**0.01**	**0.01**			
Oecomys spp.			**X**			
Potos flavus	kinkajou	**0.01**				
Proechimys spp.	spiny rat		**X**	**X**		
Pseudoryzomys simplex	Brazilian false rice rat	**X**				
Rattus spp.	rat	**0.01**	**X**			
Sciurus aestuans	Guianan squirrel					**X**
Reptiles						
Paleosuchus trigonatus	smooth-fronted caiman	**0.01**				
Ameiva ameiva	South American ground lizard	**0.01**				
Amphibians						
Leptodactylus pentadactylus	smoky jungle frog		**X**			
Fish						
Characidae (family)		**0.01**				
Birds						
Amazona spp.		**X**				
Bucco capensis		**X**				
Bucconidae (family)		**0.02**				
Buteo spp.	hawk	**0.01**				
Cairina moschata	Muscovy duck		**X**			

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Cathartidae (family)		**X**		**X**
			X	
Charadriidae (family)			**X**	**0.01**
Coragyps atratus	black vulture	**X**	**X**	
Crotophaga ani	smooth-billed ani	**X**		
Crypturellus tataupa	Tataupa tinamou	**0.01**		
Crypturellus undulatus	undulated tinamou	**0.02**	**X**	
Fringillidae (family)	finch	**0.05**	**X**	**X**
Gallus gallus	red junglefowl	**0.02**	**X**	
Lepidocolaptes spp.		**0.01**	**X**	**X**
Lepidothrix coronata	blue-crowned manakin			
Nothoprocta spp.		**0.01**		
Nyctiphrynus spp.	poorwill	**X**		
Phasiandidae (family)		**0.02**	**X**	**0.07**
Piaya cayana		**0.02**	**X**	
Psophia viridis	dark-winged trumpeter		**X**	
Ramphastos tucanus	white-throated tucan		**X**	
Rhea americana	greater rhea	**0.02**		

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Strigidae (family)		**X**		
Thamnophilidae (family)		**0.02**	**X**	**0.01**
Thraupidae (family)		**0.03**		
Tinamidae (family)		**0.02**	**X**	
Trogon viridis	green-backed trogon	**X**		
Tyrannidae (family)		**0.01**		
Vanellus spp.		**0.01**		

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Figure 1: Map of the study region centered at Sinop, Mato Grosso, Brazil (shown in purple). The area shaded in green is designated as forest with our study sites labeled as pink circles with an example of the typical landscape at a forested site shown in the photo. We sampled a total of 39 sites using UV LED CDC light traps (for sandflies and mosquitos) and 3 carrion fly traps and at 33 of these sites we also used camera traps. The inset map of Brazil shows the Amazonia biome highlighted in green with our study area highlighted in pink.

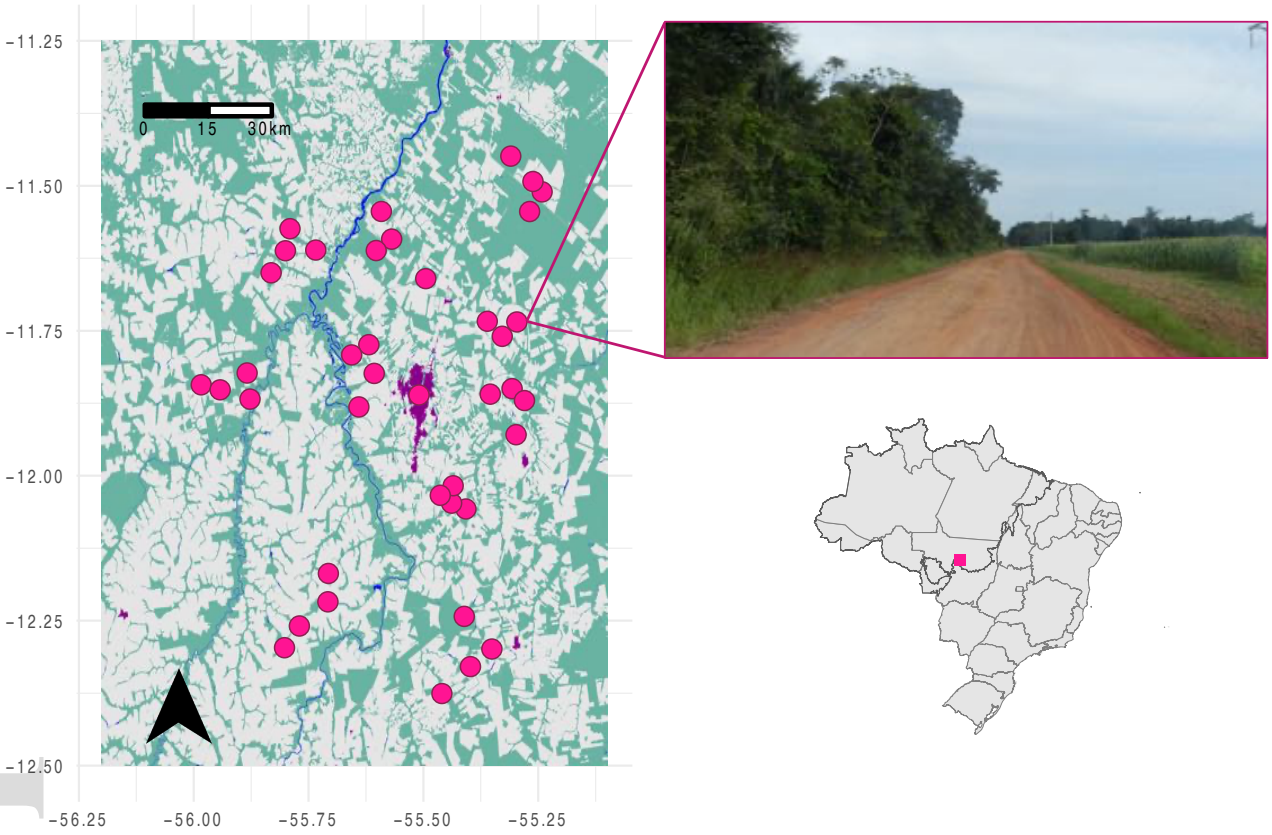

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Figure 2: Species accumulation curves based on the species diversity found with either an iDNA sampler or with camera traps. Solid lines indicate interpolation while dashed lines indicate extrapolation. (A) For the pooled insect samples, extrapolation parameters were set to 1000 pooled samples to reflect the high number of total sandfly pooled samples. A pooled sample of insects is equal to either 50 sandflies, 15 mosquitos, or 5 carrion flies that were used for DNA extraction and metabarcoding. A camera day is equal to one 24-hour period for an individual camera. The shaded region surrounding each line shows the 95% confidence intervals. (B) For sites, we compared the species richness found with the different iDNA samplers and iDNA traps (sandflies and mosquitos were trapped simultaneously using CDC UV light traps whereas carrion flies were trapped using homemade carrion fly traps). Using a combination of CDC UV light traps and carrion fly traps improves species richness at the site and landscape level compared to only carrion fly traps or only CDC UV light traps.

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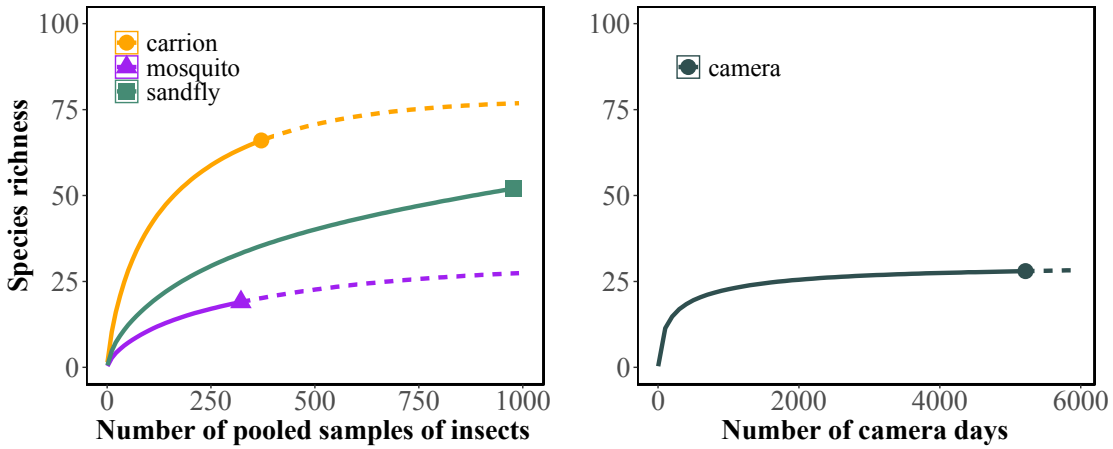

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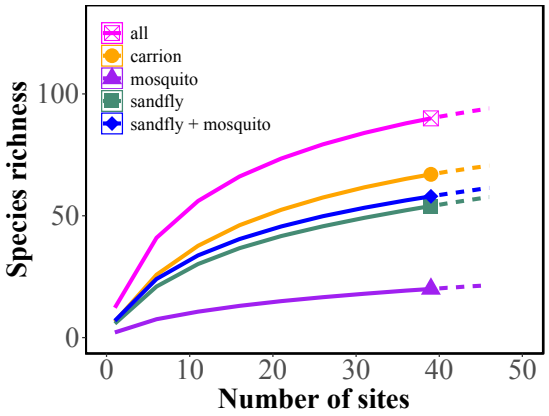

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Figure 3: Relative abundance index (RAI) for each species present within either an iDNA sampler dataset or the camera trapping dataset. Species are divided into respective groups describing their taxa or a major life history trait to compare the effectiveness of each sampling method for describing vertebrate diversity. Domestic species are often contaminants of metabarcoding, however, we found no evidence of contamination in our negative controls so this data was retained.

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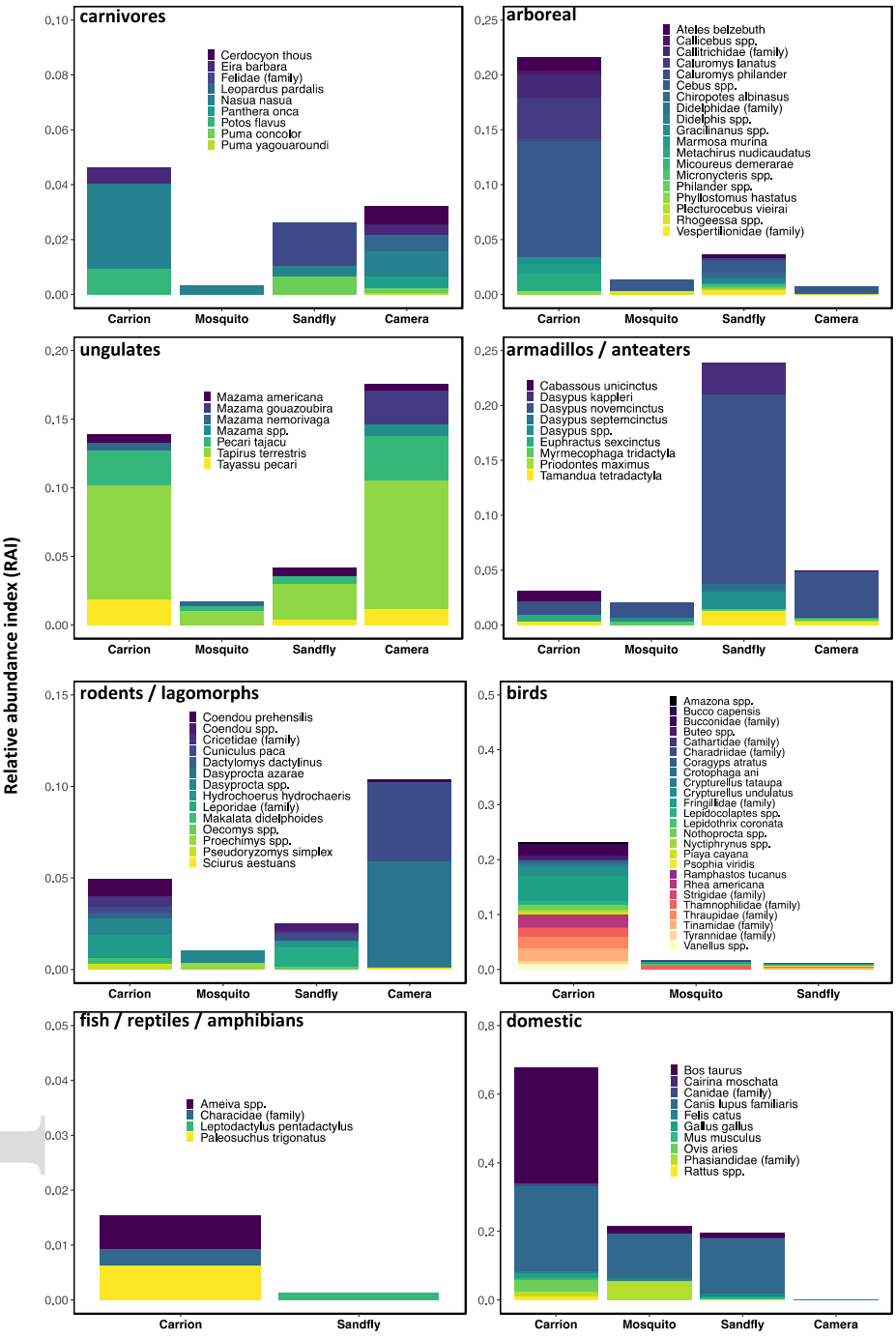

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649 **Figure 4:** The median number of species found at each site for each sampling type. Boxplots are
650 bounded by the first and third quantiles.
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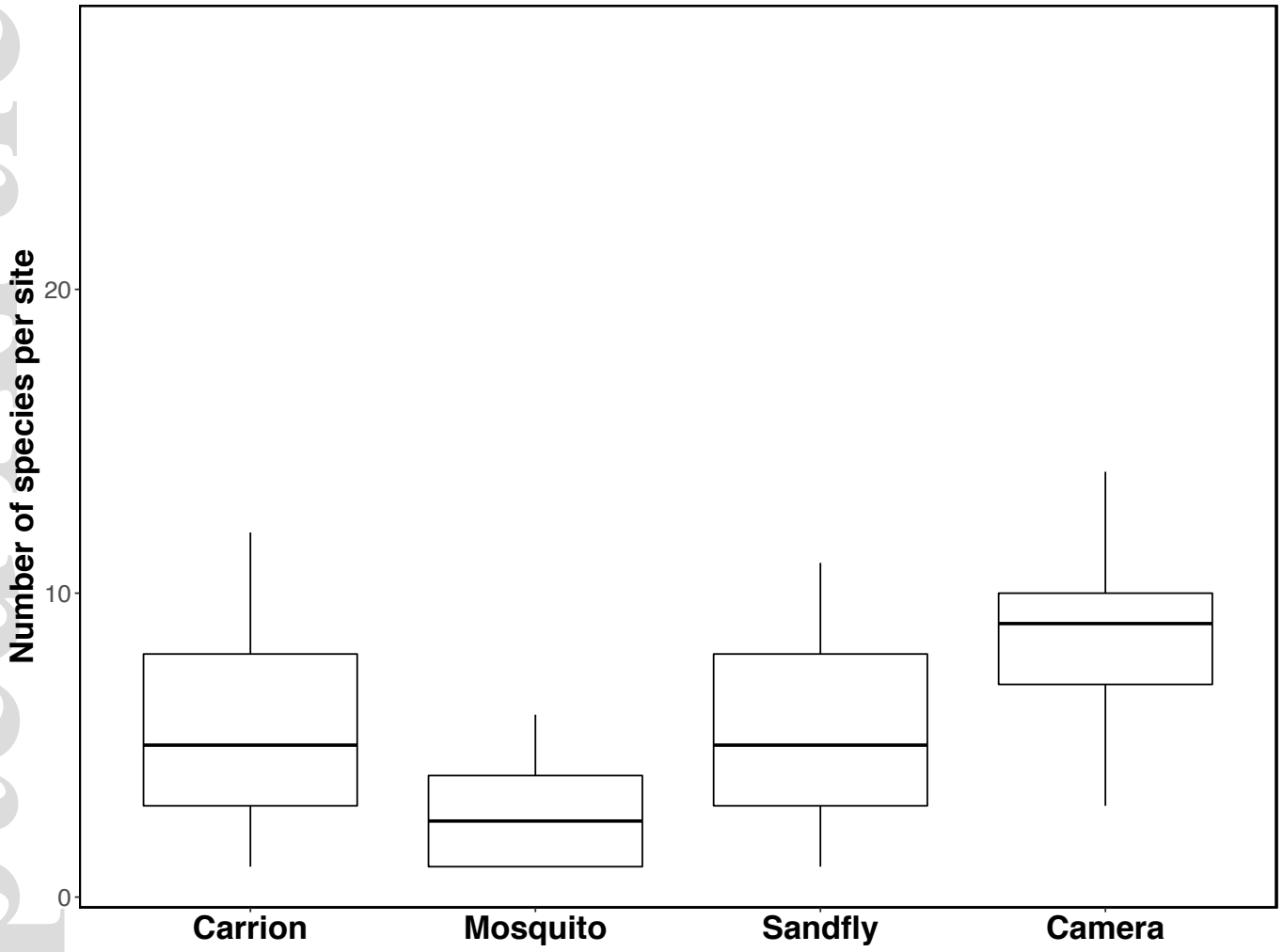

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Figure 5: NMDS ordination showing overlap in vertebrate species communities as revealed by each iDNA source. Both panels display the same ordination with (A) showing the site labels colored by the iDNA sampler and (B) showing significant species (p-value < 0.001) as vectors.

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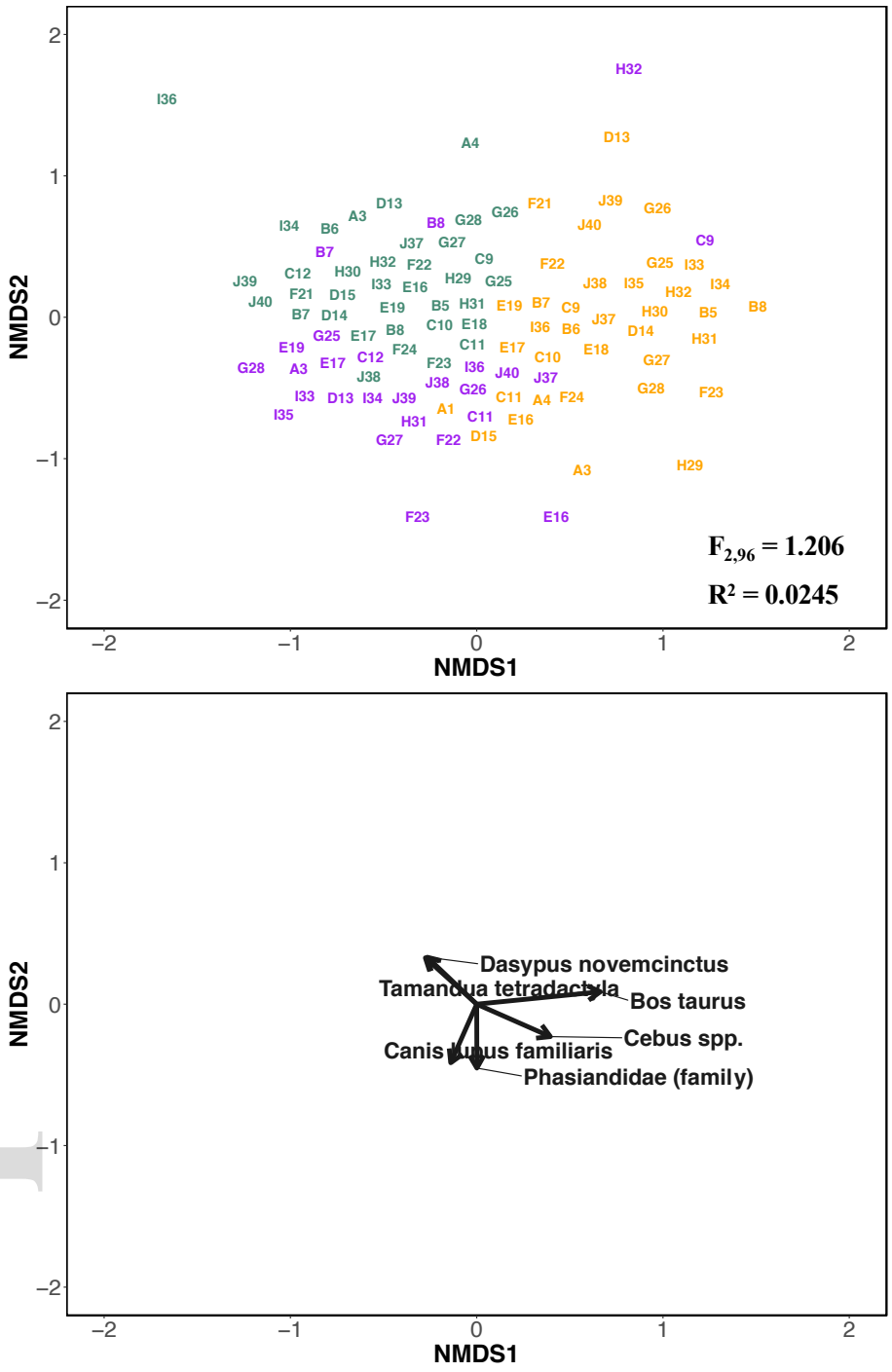

carrion
mosq
sandfly

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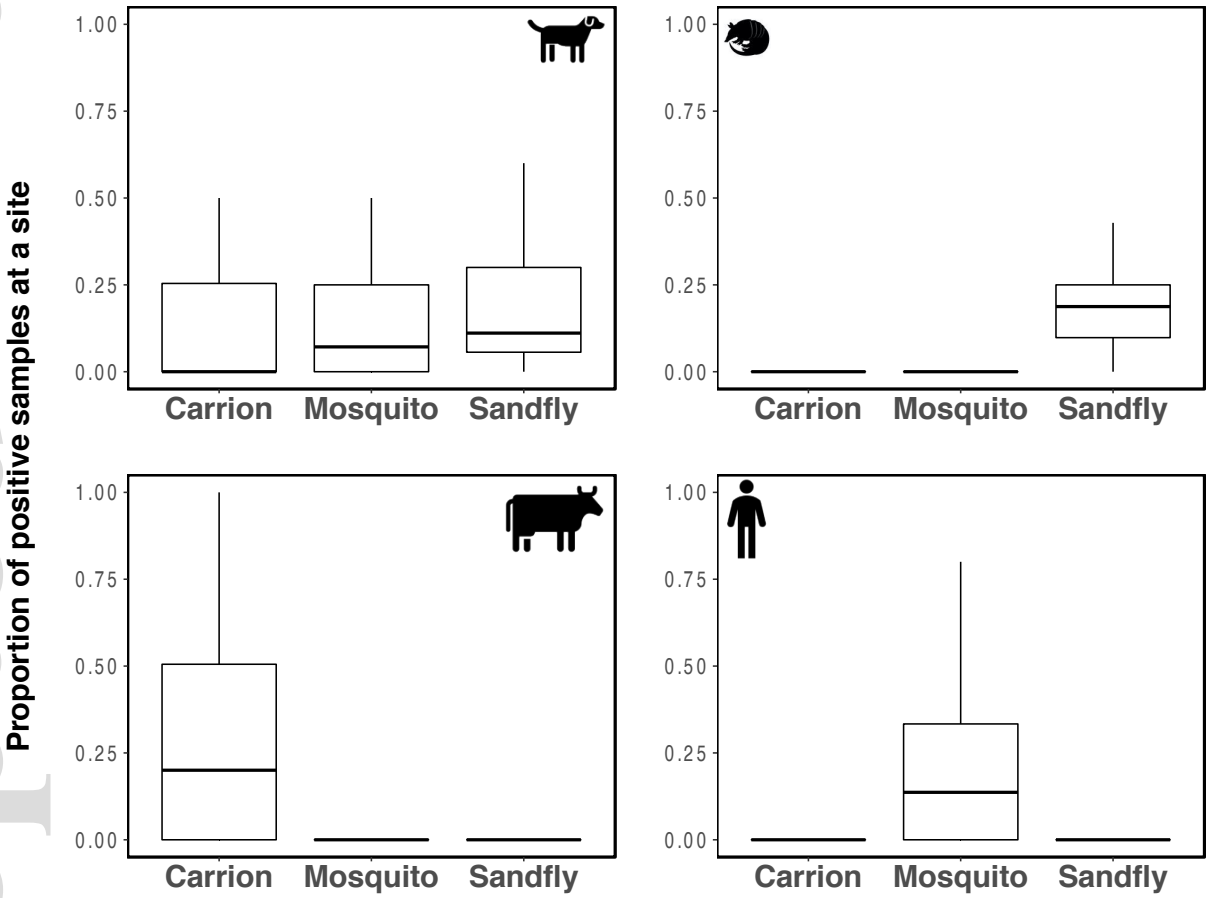
Figure 6: Proportion of dog, armadillo, cattle, and human positive samples measured at each site for each iDNA source. Data points show the proportion of total pooled samples at a site that were positive for the respective species. Domestic species, such as dog and cattle, are often contaminants of metabarcoding, however, we found no evidence of contamination in our negative controls so this data was retained.



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