

1                   **Airborne environmental DNA for terrestrial vertebrate community monitoring**

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20  
21                   **Summary**

22                   Assessing and studying the distribution, ecology, diversity and movements of species is key in  
23                   understanding environmental and anthropogenic effects on natural ecosystems. Although  
24                   environmental DNA is rapidly becoming the tool of choice to assess biodiversity<sup>1-3</sup> there are  
25                   few eDNA sample types that effectively capture terrestrial vertebrate diversity and those that  
26                   do can be laborious to collect, require special permits and contain PCR inhibitory substances,  
27                   which can lead to detection failure. Thus there is an urgent need for novel environmental DNA  
28                   approaches for efficient and cost-effective large-scale routine monitoring of terrestrial  
29                   vertebrate diversity. Here we show that DNA metabarcoding of airborne environmental DNA  
30                   filtered from air can be used to detect a wide range of local vertebrate taxa. We filtered air at  
31                   three localities in Copenhagen Zoo, detecting mammal, bird, amphibian and reptile species  
32                   present in the zoo or its immediate surroundings. Our study demonstrates that airDNA has the  
33                   capacity to complement and extend existing terrestrial vertebrate monitoring methods and  
34                   could form the cornerstone of programs to assess and monitor terrestrial communities, for  
35                   example in future global next generation biomonitoring frameworks<sup>4,5</sup>.

36  
37                   **Keywords**

38                   Air-samplers, Bioaerosol, Biodiversity, Biomonitoring, Conservation, Environmental DNA

39

40 **Main**

41 Biodiversity monitoring at the community scale is a critical element of assessing and studying  
42 species distributions, ecology, diversity and movements <sup>e.g. 6,7</sup>. Further, it informs conservation  
43 efforts, evaluates status and quotas on species subject to recreational or commercial harvest,  
44 detects the arrival of invasive species, and tracks progress in achieving biodiversity targets;  
45 crucial aims in light of the current climate and biodiversity crisis <sup>7–9</sup>. This highlights the urgent  
46 need for efficient and cost-effective methods with which to document and monitor biological  
47 communities.

48

49 Over the last decade the analysis of environmental DNA, or eDNA, has emerged as a valuable  
50 tool for non-invasive, sensitive and cost-effective characterization of biodiversity and species  
51 communities that complements and extends existing methods <sup>1–3</sup>. Typically eDNA is extracted  
52 from samples such as sediments, water, faeces or gut contents, and is a complex mixture of  
53 intra- and extracellular DNA derived from many sources and of different qualities <sup>1</sup>. DNA  
54 metabarcoding coupled with high-throughput sequencing is generally used to sequence  
55 taxonomically informative markers <sup>10</sup>. This has allowed compilation of species inventories,  
56 detection of common, rare, indicator and invasive species, and has provided information about  
57 plant-pollinator interactions and ecosystem services and dynamics <sup>e.g. 11–17</sup>. Further, there is  
58 progress towards implementation of eDNA in routine biodiversity monitoring at both local and  
59 global scales <sup>4,5,18,19</sup>.

60

61 Vertebrates are key species in most terrestrial ecosystems, but are experiencing extinctions and  
62 declines in population numbers and sizes due to increasing threats from human activities and  
63 environmental change <sup>20–23</sup>; [www.iucnredlist.org](http://www.iucnredlist.org). Terrestrial vertebrate monitoring is,  
64 however, generally expensive, laborious and difficult with existing methods, and so far,  
65 terrestrial vertebrate monitoring with eDNA has been challenged by that only few of the  
66 currently applied eDNA sample types are capable of capturing community-scale terrestrial  
67 vertebrate diversity. Two eDNA sample types dominate such analyses: freshwater samples and  
68 invertebrate gut contents. In freshwater, terrestrial vertebrates can be detected through the  
69 DNA they leave when e.g. drinking or defecating <sup>24–28</sup>, and DNA from vertebrates can be  
70 detected in the gut contents of parasitic, scavenging or coprophagous invertebrates <sup>13,29–32</sup>.  
71 However, invertebrate and freshwater samples can require permits and be laborious to collect,  
72 and may contain enzyme inhibitors such as heme compounds and humic acids which can hinder  
73 or introduce stochasticity in the metabarcoding PCR amplification of vertebrate DNA, leading to  
74 false negatives <sup>33–35</sup>. Further, they represent relatively biased samples of vertebrate DNA due to  
75 potential invertebrate feeding preferences <sup>36</sup> and bias towards terrestrial vertebrates leaving  
76 DNA in freshwater <sup>28</sup>. Hence, for eDNA-based monitoring of terrestrial vertebrates there is a  
77 gap between the operational difficulties and shortcomings of the currently established

78 substrates and the urgent need for innovative, efficient and cost-effective methods for  
79 assessing vertebrate community composition.

80

81 We hypothesised that DNA captured from the air could solve these issues, potentially allowing  
82 for straightforward collection and characterisation of community scale distribution data from  
83 terrestrial vertebrates. Air is filled with particles, such as fungal spores, bacteria, vira, pollen,  
84 dust, sand, droplets and fibrous material, which can be airborne for days and transported over  
85 long distances in the atmosphere depending on humidity and particle size <sup>37–42</sup>. These contain  
86 DNA and/or carry DNA attached to them, and recently DNA sequencing has been used to  
87 identify the taxonomic origins of airborne fungal spores, algae, pollen and microbiota collected  
88 on adhesive tape, in air filters and in dust traps <sup>43–49</sup>. Further, two studies have indicated that  
89 micro-sized tissue fragments and debris from vertebrates can be airborne and detected through  
90 DNA-sequencing. One study demonstrated vertebrate detection from DNA filters in a small,  
91 confined room containing hundreds of individuals of the target species <sup>50</sup>. Another study  
92 sequenced DNA from atmospheric dust samples in the Global Dust Belt over the Red Sea and  
93 detected eukaryotes, including small sequence quantities of human, cetacean and bird <sup>51</sup>.  
94 However, the use of airDNA for studying and monitoring local vertebrate communities in a  
95 wider context is unexplored. Here, we demonstrate that a wide range of local terrestrial  
96 vertebrate taxa can be detected by sequencing of particles filtered from air, providing a new  
97 framework for airDNA assessment of terrestrial vertebrate communities.

98

### 99 **Terrestrial vertebrates leave detectable DNA in air**

100 To investigate whether terrestrial vertebrates leave detectable DNA traces in air, we filtered air  
101 in Copenhagen Zoo, Denmark, which provided an ideal, controlled setting with a well-defined  
102 population of vertebrates exotic to the surrounding environment. Air was filtered for between  
103 30 mins and 30 hrs using three different samplers; a water vacuum using line power in which air  
104 circulated through sterile water which was then filtered using a Sterivex filter, and two air  
105 particle samplers with class F8 fibrous filters for airborne particulate matter. One used a 24 V  
106 blower fan requiring line power and to pave the way for vertebrate monitoring in the wild, the  
107 other used a 5 V blower fan with a mobile phone power bank. Each sampling was carried out in  
108 duplicate: two consecutive replicates for the water vacuum, and two simultaneous replicates  
109 for each of the samples taken with the novel particle samplers, resulting in a total of 40 samples  
110 across the three sampling locations. DNA was extracted and high-throughput sequenced at two  
111 mtDNA metabarcoding markers: one targeting vertebrates in general and the other mammals  
112 specifically <sup>52,53</sup>. In our data analysis we only retained Operational Taxonomic Units (OTU) that  
113 could be identified at species level, thereby providing a conservative inventory of vertebrate  
114 detections.

115

116 We first tested airDNA monitoring in a well-ventilated semi-confined space by collecting 12  
117 airborne particulate matter samples in a stable in the southern section of the zoo holding two  
118 okapis (*Okapia johnstoni*) and two red forest duikers (*Cephalophus natalensis*) (Fig. 1a). Using  
119 this approach, we detected both the species present in the stable in all of the 12 samples.  
120 Further, we detected 13 birds and mammals that are kept in neighbouring outdoor enclosures  
121 in the southern section of the zoo, 1 zoo animal that was located in the northern section of the  
122 zoo, 2 animals kept in the zoo but that are also known to be pests, 2 wild or domestic non-zoo  
123 species known to occur in and around the zoo, and 2 fish species used as feed in the zoo (Fig.  
124 2). Thus, overall, we detected 22 non-human vertebrate species (Fig. 2; Supplementary Table 1)  
125 with the number of species detected per sample ranging from 6 to 17 (mean = 11.33, SD= 3.17)  
126 (Supplementary Table 2).

127  
128 To further explore the potential of airDNA to monitor terrestrial vertebrate communities, we  
129 deployed air samplers at a location proximal to multiple outdoor mammal and bird enclosures  
130 in the southern section of the zoo (Fig. 1b). In total, 16 samples of airborne particulate matter  
131 were collected, split between the water vacuum (n = 4 in September; n=4 in December), and  
132 the 5 V (n=2 in December) and 24 V samplers (n=6 in December). Between 8 and 21 non-human  
133 vertebrates (mean = 14.5, SD= 4.69) were detected in each of the 16 samples (Supplementary  
134 Table 2), totalling 30 non-human vertebrate species for the outdoor sampling site (Fig. 2;  
135 Supplementary Table 3). Among these, we detected 21 of the 35 bird and mammal species that  
136 had access to an outdoor enclosure in the southern section of the zoo (Fig. 1b; Fig. 2). We  
137 further detected 1 zoo animal present in the north section of the zoo, 2 animals kept in the zoo  
138 but that are also known to be pests (i.e. house mouse and brown rat), 5 wild or domestic non-  
139 zoo mammal species known to occur in and around the zoo (e.g. cat and squirrel) and 1 fish  
140 species used as feed.

141  
142 To test whether sequencing of airborne particulate matter would allow detection of taxonomic  
143 groups other than birds and mammals, we collected 12 samples inside the Tropical House (Fig.  
144 1a). The Tropical House consists of two main parts, the Butterfly House and the Rainforest  
145 House. We sampled in the latter, which contains multiple reptile, bird, and mammal species not  
146 present in the outdoor enclosures, except for the Eurasian hoopoe (*Upupa epops*). In the 12  
147 samples collected in the Rainforest House, we detected 7 to 17 non-human vertebrate species  
148 per sample (mean = 12.17, SD = 2.98) summing to a total of 29 species, including 16 mammal, 8  
149 bird, 3 fish, 1 amphibian and 1 reptile species (Fig. 2; Supplementary Table 4; Supplementary  
150 Table 2). These 29 species included 9 of the 24 vertebrate species kept in the Rainforest House  
151 of which 1 of the detected species is kept within a terrarium, namely the Dumeril's ground boa  
152 (*Acantophis dumerili*). In addition, we detected 5 species kept in other parts of the Tropical  
153 House, 4 species used as feed in the zoo, and 7 zoo species kept outside the Tropical House.

154 Further, we detected 2 wild or domestic non-zoo species known to occur in and around the zoo,  
155 and 2 rodents known to be pests (Fig. 2). For the total list of species present in the entire  
156 Tropical House, see Supplementary Table 5.

157

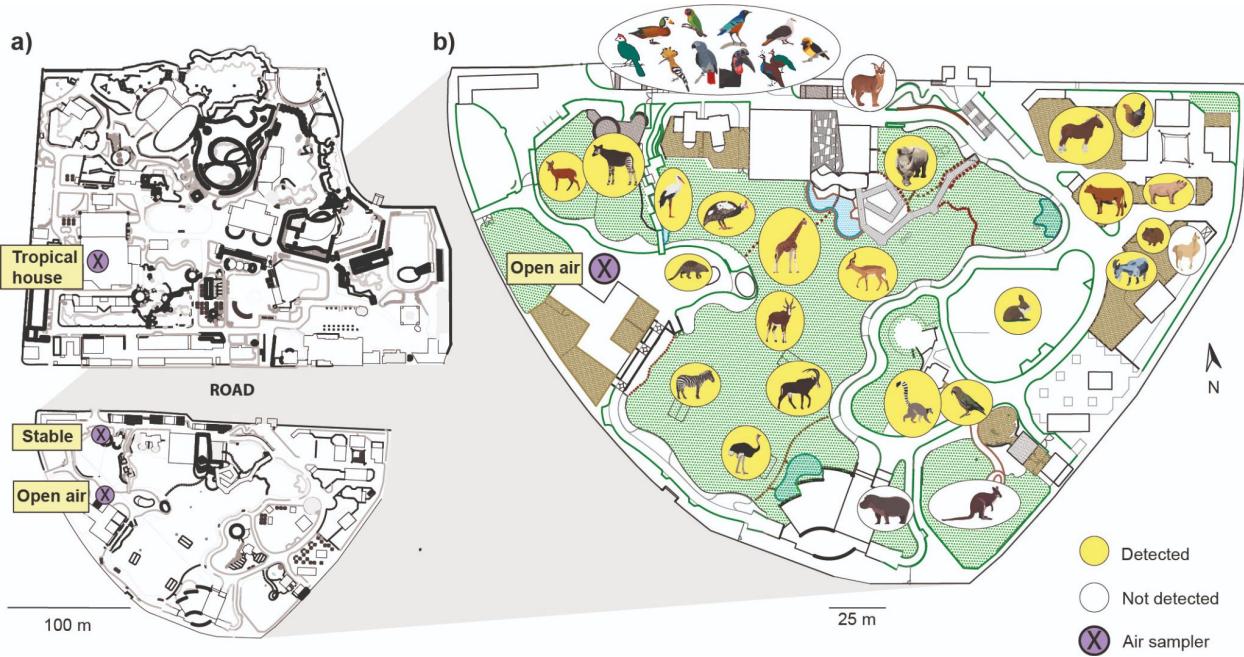
158 We collated all data across sites and samples in an overall inventory. We detected between 9  
159 and 23 non-human vertebrate species per sample (mean = 15.6, SD = 4.06), summing to a total  
160 of 49 vertebrate species spanning 26 taxonomic orders and 37 families; 30 mammal, 13 bird, 4  
161 fish, 1 amphibian and 1 reptile species (Fig. 2). Of these 49 species, 38 were exotic animals kept  
162 in the zoo, 3 were fish species routinely used as animal feed in the zoo, 2 rodent species kept at  
163 the zoo but also known to be pests, and the remaining 6 were wild or domestic non-zoo species  
164 known to occur in or around the zoo. Thus, the presence of all 49 detected species could be  
165 accounted for. The robustness of our method is further demonstrated by 39 matching species  
166 detections between the two sets of sampling replicates, with the remaining 10 taxa only being  
167 detected by one of the two sampling replicate sets. These are conservative identifications as  
168 they only include those OTU sequences that could be identified to species level. However, for  
169 OTU sequences that we could only assign to higher taxonomic levels, we detected Columbidae,  
170 a bird family consisting of pigeons and doves, Passeriformes, a large song-bird family, and  
171 *Corvus* sp, corvids. These taxonomic groups include wild or feral birds such as jackdaws, crows,  
172 pigeons and house sparrows, which are common in and around the zoo.

173

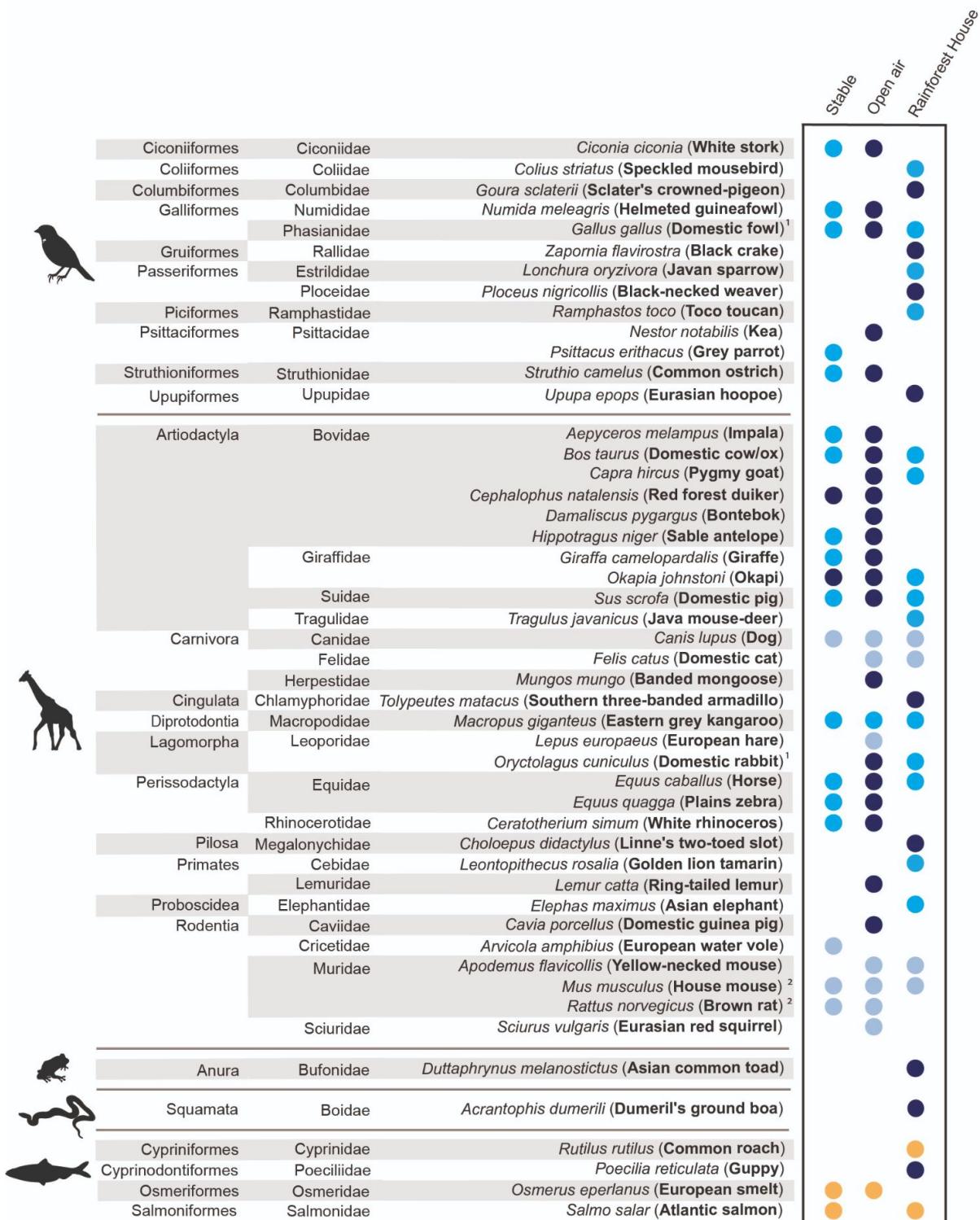
174 The detected vertebrates represent species with a large variation in sizes, behaviours and the  
175 number of animals present in the zoo, illustrating that a wide range of species can be detected  
176 by airDNA sampling. For example, among the species we detected, the zoo holds 2 ostriches  
177 (*Struthio camelus*) each weighing ca. 90 kg, 5 white rhinoceros (*Ceratotherium simum*) each  
178 weighing ca. 1800 kg, 25 helmeted guineafowls (*Numida meleagris*) each weighing ca. 1.3 kg,  
179 and 47 Javan sparrows (*Lonchura oryzivora*) each weighing ca. 22 g. Furthermore, although  
180 most of the detected vertebrate species were cursorial (e.g. the impala, *Aepyceros melampus*;  
181 and the Java mouse-deer, *Tragulus javanicus*), other lifestyles were also detected, including  
182 volant birds (e.g. kea, *Nestor notabilis*), a crawling snake (Dumeril's ground boa, *Acrantophis  
183 dumerili*) and arboreal animals (e.g. two-toed sloth, *Choloepus didactylus*).

184

185



186  
187 **Figure 1. The sampling sites and airDNA detections of vertebrate species.** a) The three  
188 locations where airDNA samples were collected in Copenhagen Zoo, Denmark: the okapi and  
189 red forest duiker stable, in open air among the outdoor enclosures and inside the tropical  
190 house. b) AirDNA sampling in open air. Visualised vertebrates have access to outdoor  
191 enclosures in the southern part of the zoo. Vertebrate species detected through DNA  
192 metabarcoding of airDNA are highlighted in yellow. Maps and animal illustrations courtesy of  
193 Copenhagen Zoo.



- Detected zoo animals kept in sampling location
- Detected zoo animals kept in a different location within the zoo
- Detected non-zoo animals occurring in or around the zoo
- Detected animals used only for feed

<sup>1</sup> Zoo animal also used as feed

<sup>2</sup> Zoo animal also found in and around the zoo as a pest

195 **Figure 2. Vertebrate species detected through metabarcoding of airDNA.** Detections are made  
196 through DNA metabarcoding of 40 samples of airborne particles from three sampling locations  
197 in Copenhagen Zoo, Denmark: the okapi and red forest duiker stable (n=12), outside among the  
198 outdoor animal enclosures (n=14) and inside the Rainforest House within the Tropical House  
199 (n=12). Only taxa that could be determined to species level are included. Taxonomic order and  
200 family are listed for each species; common names are in bold. Detected species fall within four  
201 categories; detected through air DNA sampling where they are kept (dark blue), detected in  
202 another sampling location than where they are kept (blue), detection of wild or domestic non-  
203 zoo species (light blue), and species used as animal feed (orange). Some animals kept at the zoo  
204 (domestic rabbit and fowl) were also used for feed (1). Further, other animals kept at the zoo  
205 (house mouse and brown rat) are known to occur as pests in and around the zoo (2). Detections  
206 were made with DNA metabarcoding with two mitochondrial primer sets, one targeting a  
207 mammal and one targeting a vertebrate marker. Animal illustrations obtained from the  
208 Integration and Application Network ([ian.umces.edu/media-library](http://ian.umces.edu/media-library)).  
209

#### 210 **Biomass and distance to air sampling device influence detection**

211 In studies of natural systems, airDNA will predominantly be collected in open air. Thus, we  
212 explored putative factors influencing the detection of vertebrate DNA in the outdoor sampling  
213 site. This included comparing average biomass and distance from sampler for the species we  
214 detected versus those not detected. In addition, we used a logistic regression model with air  
215 filtering method, sampling time, average distance of animal to the samplers, animal biomass  
216 (no. individuals x average weight for individuals, log transformed) and the taxonomic group  
217 (mammal and bird) as independent variables. We found that higher animal biomass (p-value <  
218 0.001) (Fig. 3b) and a shorter distance to the sampler (p-value < 0.05) (Fig. 3c) and significantly  
219 increased the probability of vertebrate DNA detection, but found no significant effect of the  
220 taxonomic class, the choice of sampling device or sampling time. We hypothesise that larger  
221 animals shed more DNA and are more easily detected, per individual. However, when excluding  
222 biomass from the model, mammals had a higher probability of being detected than birds (p-  
223 value < 0.001).  
224

225 The concentration of DNA is expected to fall with distance from the source, and accordingly we  
226 only detected one of the species located in the northern section of the zoo, namely the eastern  
227 grey kangaroo (*Macropus giganteus*). We speculate that distance and the presence of several  
228 buildings and a trafficked road between our sampling site in the southern part of the zoo and  
229 the northern part prevented us from detecting other vertebrate species present in the northern  
230 part of the zoo. Similarly, the failure to detect 14 species present in the southern part of the  
231 zoo could not only be due to the biomass, distance and taxonomic groups, but also the  
232 presence of buildings between the enclosure and the air sampler (Fig. 1b

233 Despite not finding significant differences in detections between samplers, we did observe  
234 practical differences. We found the water vacuum sampler to be more noisy and less flexible  
235 due to its size and the need for an external power supply and molecular grade sterilized water.  
236 In contrast, the two particle samplers have the advantage of being small and portable, which  
237 allows them to be deployed in a wide variety of environments for several days depending on  
238 the power supply. The versatility comes at the expense of airflow, as the particle filter sampler  
239 with the 24 V blower fan provides a larger airflow of about 0.8 m<sup>3</sup>/min compared to the particle  
240 filter sampler with the 5 V blower fan with about 0.03 m<sup>3</sup>/min. Nevertheless, the compact size  
241 of both samplers and their very low noise level (the 24 VDC blower fan is rated at 55 dB-A)  
242 makes them suitable for environments where wildlife is easily disturbed. As it can be assumed  
243 that wildlife-DNA will often travel in association with airborne dust and fibers, typically in the  
244 size range of 1 µm-10 µm, using a less dense filter than the F8 used herein, would both  
245 decrease the collection efficiency towards smaller particles and increase the airflow through  
246 the filter. It is therefore necessary to consider the product of the two (collection efficiency \*  
247 airflow) when calculating the effective sampling volume.

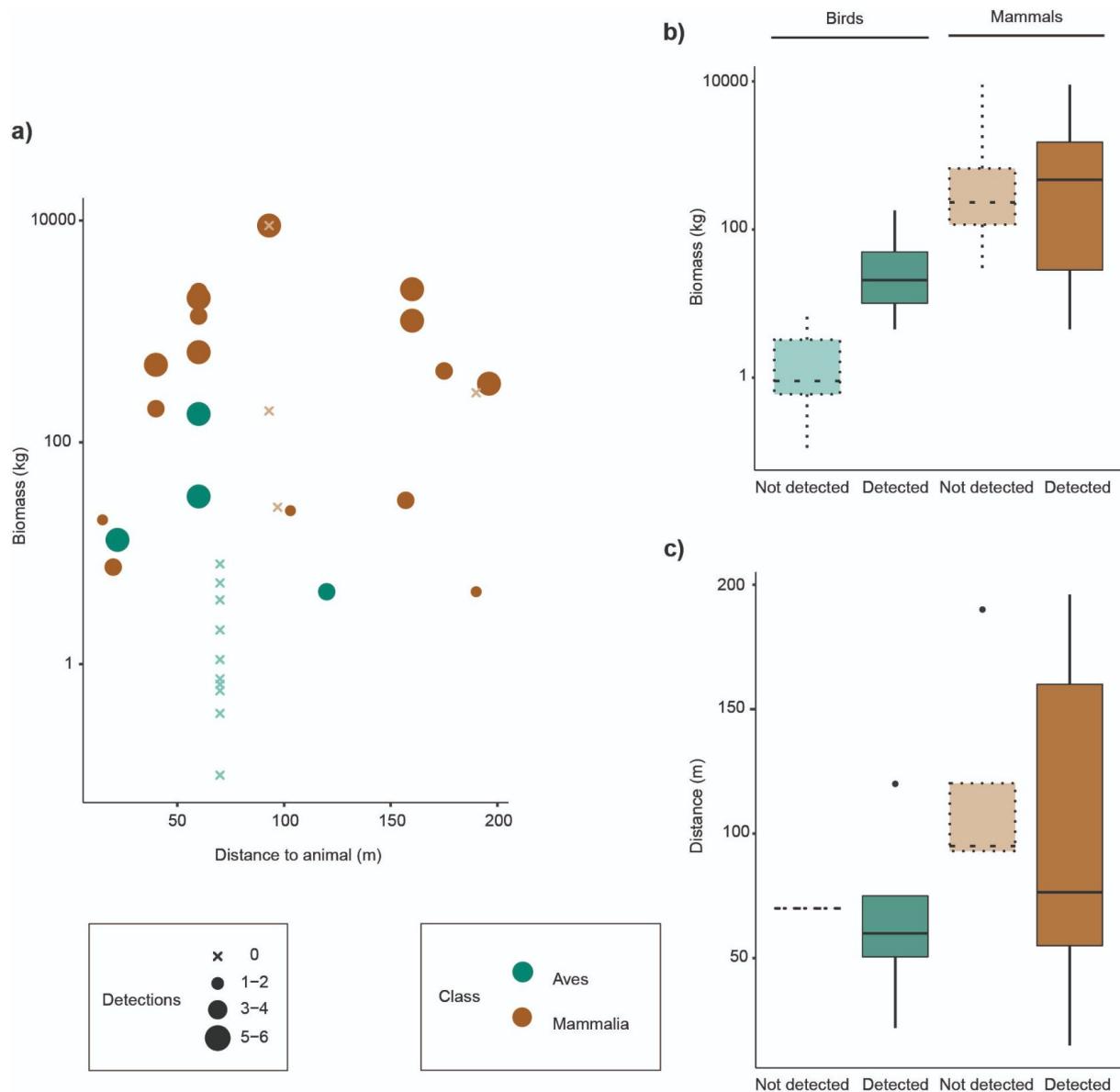
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254 **Fig. 3. Factors influencing airDNA detections of vertebrate species in open air.** The effect of  
255 biomass, distance on species detections. Only data from zoo animals with access to an outdoor  
256 enclosure in the southern part of the zoo, whether they were detected or not. a) Influence of  
257 biomass and distance on the number of times a species was detected across the six different  
258 sampling events (i.e. water vacuum for 30 and 60 min, particle filter sampler with the 24 V  
259 sampler ran during 30, 60 and 300 min, and the 5 V sampler ran during 30 hrs). b) Average  
260 biomass estimated as weight times the number of animals for the bird and mammal species  
261 that were detected and not detected in any of the air samples. c) Average distance between the  
262 samplers and open air enclosures for the bird and mammal species that were detected and not  
263 detected in any of the air samples.

264

265

266 **Implications for monitoring of terrestrial vertebrate communities**

267 Our results suggest that airDNA is an untapped source of spatial and temporal vertebrate  
268 distribution data with the potential to transform the way natural ecosystems are studied and  
269 surveyed. This includes acting as a cost-effective and efficient tool to inform conservation  
270 efforts, evaluate sustainable removal levels, and track progress in achieving biodiversity targets,  
271 something of great global importance given the ongoing climate and biodiversity crisis <sup>7–9</sup>.

272

273 We carried out the study at the Copenhagen Zoo which provided a suitable controlled source  
274 due to the presence of well-defined individual animals. However, their confinement and density  
275 in the enclosure may have artificially increased their probability of being detected in air samples  
276 compared to sampling in a natural environment. Still, we detected six non-zoo animals in the air  
277 samples despite the high zoo species biomass and concentration as compared to non-zoo  
278 animals in the surrounding area.

279

280 As with any novel methodology, including the first demonstrations of eDNA in aquatic  
281 environments <sup>15,54,55</sup>, the full potential of airDNA for vertebrate community surveys will require  
282 further optimisations and developments across a range of natural habitats and applications  
283 before standardisation and implementation in routine monitoring can be achieved. With time,  
284 we envision terrestrial airDNA vertebrate surveys could parallel the field of aquatic eDNA  
285 monitoring with the potential to revolutionize and form the cornerstone in future ecosystem  
286 studies, including global next generation biomonitoring frameworks <sup>4,5</sup>

287

288 **Competing interests**

289 Matthew Johnson is the Chief Science Officer at Airlabs, the company that designed and built  
290 the 3D housings for the particle samplers used in this study. The blueprints of these housings  
291 are however freely available and provided in Supplementary Methods. All other air sampling  
292 equipment, i.e. the water vacuum, blower-fans used for the 24 V and 5 V particle samplers, and  
293 the batteries are available in commercial companies not related to Airlabs. Thereby, the current  
294 study is not of direct commercial value to Airlabs. None of the remaining authors had any  
295 conflicts of interest.

296

297 **Author contributions**

298 CL, MTO and KB conceived and designed the study with input from CVJ, MFB and MSJ; MTO and  
299 KB provided funding; CL, CVJ and MSJ designed and tested the particle samplers; CL performed  
300 the sampling and the lab work; CL, MFB, TGF, MTO and KB analysed the data; CL, CVJ, MTO and  
301 KB drafted the paper. All authors read and approved of the final version.

302

303 **Acknowledgments**

304 This study was funded by an Experiment grant (grant no. 00028049) from VILLUM FONDEN to  
305 MTO and KB. We thank Ruta Sidaraviciute from Airlabs for helping with the design and 3D-  
306 printing of the particle samplers. We thank the Copenhagen Zoo staff for facilitating the sample  
307 collection and Frederik Leerhøi, Physilia Chua, Åshild Vågene, Sarah Mak, Jonas Niemann, and  
308 Camilla Pereira for assistance with sample collection. We thank Kurt Kjær, Fabian Roger, Lasse  
309 Vinner and Mette Juul Jacobsen for discussions, and Hannes Schoeder and Tom Gilbert for  
310 comments on the original manuscript.

311

## 312 **Methods**

### 313 Study site

314 Fortyfour air samples were collected at the Copenhagen Zoo, Denmark, during September and  
315 December 2020. Air samples were collected in three places: 1) inside a 155 m<sup>2</sup> stable inhabited  
316 by two okapis (*Okapi johnstoni*) and two red duiker (*Cephalophus natalensis*), which had the  
317 option to also use an adjoining outdoor enclosure during the day, 2) we sampled outdoors in  
318 open air at a fixed location in the part of the Zoo containing multiple outdoor enclosures with a  
319 mixed variety of mammals and other terrestrial vertebrates. Finally, 3) inside the Rainforest  
320 House, a 442 m<sup>2</sup>/2200 m<sup>3</sup> confined enclosure in which smaller vertebrates and other animals  
321 from the tropics move freely around both day and night located within the Tropical House (Fig  
322 1; Supplementary Table 5). Inside the stable and the Rainforest House, the temperature was  
323 kept constant during September and December, ranging from 18.6 to 20.5°C, and from 22 to  
324 27°C, respectively. None of the locations were directly exposed to the wind, but the stable did  
325 have openings to the outdoors and Rainforest House had an internal ventilation system. During  
326 the outdoor sample collection, on the 11th of September the temperature ranged from 17.1 to  
327 17.3°C, wind speed from 4.4 to 5.2 m/s with wind coming from the SW; on the 22th of  
328 September the temperature ranged from 21 to 21.2 °C, wind speed of 2.6 to 4.5 m/s and with  
329 wind coming from S; on the 10th of December, the temperature was 3°C, wind speed was 5.9 to  
330 5 m/s and the wind came from the East; and on the 11th of December, the temperature was  
331 2.4°C, wind speed 5.6 to 5.4 m/s and the wind came from SE/E direction (dmi.dk).

332

### 333 AirDNA samplers

334 Environmental DNA was collected from air using three different samplers, a water-based  
335 commercial vacuum and two air particle filters with different power sources and airflows. The  
336 water-based commercial vacuum was the Kärcher DS5800 Water Vacuum (WV) (Alfred Kärcher  
337 GmbH & Co. KG, Germany), which consists of a high-flow-rate impinger with an outer part that  
338 creates suction and an inner vortex chamber where the particles flow into <sup>56</sup>. This WV was  
339 connected to the electrical circuit and provided an average airflow of 8.8 m<sup>3</sup>/min.

340

341 The second sampler was a custom made air particle filter sampler consisting of a Delta  
342 Electronics 97.2 mm x 33 mm 24 V, 0.550 A DC brushless radial blower fan, a class F8 pleated  
343 fibrous particulate filter (Dongguan Wonen Environmental Protection Technology Co.,Ltd), and  
344 a 3D-printed filter housing (Airlabs, Copenhagen, Denmark; 3D-printing blueprints available in  
345 Supplementary Information). The filter was placed approximately 40 mm from the intake of the  
346 blower fan and was connected to the electrical circuit, providing an airflow of 0.8 m<sup>3</sup>/min. We  
347 call this the medium filter (MF) sampler.

348

349 The third sampler was overall similar to the MF sampler, except that the filter was placed on a  
350 3D-printed hilter housing approximately 20 mm from the intake of the blower fan, which is a  
351 battery-driven Hawkung/Long Sheng Xin 40 mm x 40 mm x 10 mm, 5 V, 0.10 A DC brushless  
352 radial blower fan, providing an airflow of 0.03 m<sup>3</sup>/min (3D-printing blueprints available in  
353 Supplementary Information). We call this the small filter (SF) sampler. For both MF and SF  
354 samplers, we used class F8 pleated fibrous particulate filters (Dongguan Wonen Environmental  
355 Protection Technology Co.,Ltd). This type of filter is usually implemented in A/C units and is  
356 designed to capture airborne particulate matter and micro- and nanofibers with high efficiency  
357 and low pressure drop. As the filter is cut and stretched out to a single layer around the size of  
358 the filter housing, the airflow and retention efficiency is expected to decrease slightly from the  
359 official F8 rating (<https://www.emw.de/en/filter-campus/filter-classes.html>).

360

### 361 Sample collection

362 Sampling with the WV sampler followed <sup>56</sup>, i.e. the inner vortex chamber was filled with 1.7 L  
363 sterile Milli-Q H<sub>2</sub>O. After running the impinger, the water from the vortex chamber was filtered  
364 using Sterivex filters (pore size 0.22 µm). In between samplings, the vortex chamber and the  
365 suction hole were cleaned with 5% sodium hypochlorite (bleach) and 70% ethanol. At every  
366 location, a sampling negative control consisting of 200 mL of sterile Milli-Q H<sub>2</sub>O was added to  
367 the vortex chamber and thereafter filtered with Sterivex filters. Using this sampler, air was  
368 collected for 30 min and 60 min at each site during December. Samples collected outside with  
369 the WV were also collected during September. Prior to sampling with the MF and SF air filter  
370 samplers, the F8 filters were cut into a smaller size to fit the housing, autoclaved, placed under  
371 UV light for 20 min and thereafter stored individually in sterile plastic bags. In between  
372 sampling events, the housing of the MF and SF samplers was cleaned with 5% bleach and 70%  
373 ethanol. Sterilized filters were handled using sterile tweezers and stored in a sterile 50 mL  
374 Falcon tube upon sampling. To test the effect of sampling time, the MF sampler was left  
375 running for 30 min, 60 min and 5 hrs. For the SF sampler, to test the effect of long sampling  
376 time, this sampler was left running for 30 hrs at each location. Both samplers were run during  
377 December. For all three samplers, samples were taken at 1 m above the ground and in

378 duplicates. Filters were stored in a cooling box for up to 5 hours and thereafter at -20 °C until  
379 DNA extraction.

380

381 **DNA extraction**

382 Due to their big size, the MF filters were cut in half with sterile blades. Both halves of MF filters  
383 and entire SF filters were transferred independently to 5 mL Eppendorf tubes and 3 mL of  
384 autoclaved PBS pH 7.4 (1X) (Gibco™, Thermo Fisher) was added. After an incubation of 45 mins,  
385 the filters were transferred to a new Eppendorf tube, and the PBS was centrifuged at 6000 xg  
386 during 10 min to create a pellet, and the supernatant removed. The DNeasy Blood & Tissue Kit  
387 (Qiagen, USA) was used for DNA extraction of the PBS pellets, the two halves of the MF filters,  
388 the entire SF filters and the Sterivex filters from the VW sampler. In addition, to test for  
389 contamination in the sterilized MF and SF filters, Sterivex filters and the autoclaved PBS, non-  
390 used filters and PBS were subjected to DNA extraction. In addition, to test for contamination in  
391 the DNA extraction room, two falcon tubes containing 50 mL sterile Milli-Q H2O left open for 48  
392 hrs, and were also subjected to DNA extraction.

393

394 The DNA extraction followed manufacturer's instructions (Purification of Total DNA from  
395 Animal Tissues protocol), with slight modifications: the ratio 9:1 of ATL buffer to Proteinase K  
396 was kept but the volume was increased to 720 ATL and 80 Proteinase K and an incubation step  
397 of 37 °C for 15 min was added after the addition of 50 µl of EBT (EB buffer with 0.05% Tween-20  
398 (VWR)). This elution step was carried out twice to increase DNA yield.

399

400 As belonging to the same sample, the digest of the filters and from the PBS pellet were passed  
401 through the same spin column and therefore having one DNA extract per sample. However, for  
402 three MF samples collected inside the Okapi stable, the digests of each half of the filter  
403 presented many particles clogging the spin column and therefore the digests could not be  
404 combined into one spin column. This resulted in a total of 49 DNA extracts, representing 40  
405 samples (see Supplementary Table 7). Negative extraction controls were added for every 16  
406 samples. Eluted DNA was stored in Eppendorf LoBind tubes at -20°C.

407

408 To minimize contamination risk during DNA extraction, we set up a specialised environmental  
409 DNA pre-PCR laboratory, which was thoroughly cleaned prior to its use and in which guidelines  
410 follow those used in ancient DNA laboratories such as the use of hair net, sleeves, facemask,  
411 two layers of medical gloves, dedicated footwear and the use of ≥3% bleach on all surfaces<sup>57</sup>.

412

413 **DNA metabarcoding**

414 Metabarcoding was conducted using two different primer sets. To target mammals, a ca. 95 bp  
415 16S rRNA mitochondrial marker was PCR-amplified with the primers 16Smam1 (forward 5'-

416 CGGTTGGGTGACCTCGGA-3') and 16Smam2 (reverse 5'-GCTGTTATCCCTAGGGTAAC-3')  
417 (Taylor, 1996). To target vertebrates, a ca. 97 bp fragment of the 12S gene was PCR-amplified  
418 with the primer set 12SV05 forward 5'-TTAGATACCCACTATGC-3' and 12SV05 reverse 5'-  
419 TAGAACAGGCTCCTCTAG-3' (Riaz et al., 2011). The two metabarcoding primer sets are from  
420 here on referred to as 16S mammal and 12S vertebrate primers, respectively. Nucleotide tags  
421 were added to the 5' ends of forward and reverse primers of both primer sets to allow parallel  
422 sequencing <sup>58</sup>. Nucleotide tags were six nucleotide tags in length and had min. 3 nucleotide  
423 differences between them. One to two nucleotides were added to the 5' end to increase  
424 complexity on the flowcell. DNA extracts from fin whale (*Balaenoptera physalus*) and bowhead  
425 whale (*Balaena mysticetus*) were used as positive controls, as none of the species are found  
426 close to the sampling site in Copenhagen Zoo.

427  
428 Prior to metabarcoding PCR amplification, dilution series of a subset of the DNA extracts were  
429 screened using SYBR Green quantitative PCR (qPCR). This was done to determine the optimal  
430 cycle number and DNA template volume to ensure optimal amplification in the following  
431 metabarcoding PCR amplifications. Further, all negative controls were included in the qPCR to  
432 screen for contamination.

433  
434 For the 16S mammal primer, the 20  $\mu$ l reactions consisted of 2 or 4  $\mu$ l DNA template, 0.75 U  
435 AmpliTaq Gold, 1x Gold PCR Buffer, and 2.5 mM MgCl<sub>2</sub> (all from Applied Biosystems); 0.6  $\mu$ M  
436 each of 5' nucleotide tagged forward and reverse primer; 0.2 mM dNTP mix (Invitrogen); 0.5  
437 mg/ml bovine serum albumin (BSA, Bio Labs); 3  $\mu$ M human blocker (5'-3'  
438 GCGACCTCGGAGCAGAACCC-spacerC3) <sup>59</sup>; and 1 $\mu$ L of SYBR Green/ROX solution [one part SYBR  
439 Green I nucleic acid gel stain (S7563) (Invitrogen), four parts ROX Reference Dye (12223-012)  
440 (Invitrogen) and 2000 parts high-grade DMSO]. The thermal cycling profile was 95°C for 10 min,  
441 followed by 40 cycles of 95°C for 12 s, 59°C for 30 s, and 70°C for 25 s, followed by a  
442 dissociation curve. For the 12SVert primer, the 20  $\mu$ l reaction was the same except for the  
443 human blocker (5'-3' TACCCCACTATGCTTAGCCCTAACACCTAACAGTTAACATC-spacerC3) <sup>32</sup> and  
444 the thermal cycling profile of 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 59°C for 45  
445 s, and 72°C for 60 s, followed by a dissociation curve. The amplification plots from the qPCR  
446 indicated that 2  $\mu$ l DNA template was optimal, 35 and 38 cycles were optimal for the 16S  
447 mammal and 12S vertebrate primers, respectively, and the negative extraction controls showed  
448 no contamination.

449  
450 For the metabarcoding PCR, the 20  $\mu$ L reactions were set up as described for the qPCR above  
451 but omitting SYBR Green/ROX and replacing the dissociation curve with a final extension time  
452 of 72°C for 7 min. Four tagged PCR replicates were carried out for each of the 49 DNA extracts,

453 negative and positive controls, and for both primer sets; PCR replicates from each sample were  
454 differently tagged. Negative controls were included every seven PCR reactions.

455

456 Amplified PCR products were visualized on 2% agarose gels with GelRed against a 50 bp ladder.  
457 All negative controls appeared negative and all positive controls showed successful  
458 amplification. Even if not showing a successful amplification, all PCR products of DNA extracts,  
459 including negative and positive controls carrying different nucleotide tag combinations, were  
460 pooled resulting in four amplicon pools: one pool per replicate.

461

462 Amplicon pools were purified with MagBio HiPrep beads (LabLife) using a 1.6x bead to amplicon  
463 pool ratio and eluted in 35 µL EB buffer (Qiagen). Purified amplicon pools were built into  
464 sequence libraries with the TagSteady protocol to avoid tag-jumping (Carøe & Bohmann, 2020).  
465 Libraries were purified with a 1.6x bead to library ratio and eluted in 30 µL EB buffer and qPCR  
466 quantified using the NEBNext Library Quant Kit for Illumina (New England BioLabs Inc.). Purified  
467 libraries were pooled equimolarly according to the qPCR results and sequenced at the  
468 GeoGenetics Sequencing Core, University of Copenhagen, Denmark. Libraries were sequenced  
469 using 150 bp paired-end reads on an Illumina MiSeq sequencing platform using v3 chemistry  
470 aiming at 30,000 reads per PCR replicate.

471

## 472 **Data analysis**

473 Sequence data for each primer set was processed separately. Illumina adapters and low quality  
474 reads were removed and paired ends merged using AdapterRemoval v2.2.2<sup>60</sup>. Within each  
475 amplicon library, sequences were sorted based on primers and tag sequences using Begum<sup>61</sup>  
476 allowing two primer-to-sequence mismatches. Further, for each sample Begum was used to  
477 filter sequences across the PCR replicates guided by the positive and negative controls and  
478 retaining sequences found in three out of the four PCR replicates and with a minimum copy  
479 number of 10 and 6 for the 16S mammal and 12S vertebrate primer sets, respectively. As the  
480 aim of the present study was to detect and identify species and not intraspecific variation, we  
481 decided to create clusters of sequences, instead of denoising and creating amplicon sequence  
482 variants (ASV). Clustering and denoising have proven to be complementary, instead of  
483 alternatives, and when working with eukaryotes, clustering should be the standard unit as long  
484 as using the correct parameter settings during data analysis<sup>62</sup>. The filtered sequences with a  
485 similarity score of 97% were therefore clustered into operational taxonomic units (OTU) using  
486 SUMACLUST<sup>63</sup>. Curation of the OTUs was carried out with the LULU algorithm<sup>64</sup>, using default  
487 settings to remove erroneous OTUs.

488

489 Taxonomic identification of the OTU sequences was carried out using BLASTn against the NCBI  
490 Genbank database. The output was imported into MEGAN Community Edition v6.12.7<sup>65</sup> using a

491 weighted LCA algorithm with 80% coverage, top percent of 10, and a minimum score of 150.  
492 The taxonomic identification of all OTU sequences was manually checked to validate them and  
493 species-level identification was assigned if the OTU sequence had a 100% identity match to a  
494 NCBI reference sequence. We assigned those that matched 100% to more than one species to  
495 the species found in the Copenhagen Zoo. In a few cases where multiple OTUs were assigned to  
496 the same species, the corresponding DNA sequences were checked visually in Geneious Prime  
497 2020.1.2 to assess whether the OTUs resulted from genuine haplotype variation or biases  
498 caused by minor variations in sequence length. OTUs that could not be identified to species  
499 level were discarded before further analysis. In addition, sequences matching to human were  
500 removed, as well as those matching to chimpanzee (*Pan troglodytes*) due to its close similarity  
501 to human sequences. DNA from the Slater's crowned pigeon (*Goura sclateri*) was detected in  
502 the water vacuum samples collected at the open-air location, but as it was also detected in the  
503 sampling negative control, it was considered cross-contamination and therefore deleted from  
504 the data from that site. One of the few non-detected mammals in the outdoor sampling was  
505 wallaby, whereas the Eastern grey kangaroo was detected, even though it is found on the North  
506 part of the zoo. Both animals belong to the same genus, *Macropus*, but both markers show a  
507 100% match to kangaroo DNA. Finally, the OTU taxonomically identified as *Canis lupus* could  
508 originate from dog or grey wolf. Although three grey wolves were present in the zoo during the  
509 sampling in September, they were absent during the sampling in December. Further, the  
510 detection of this OTU in all samplers made us conclude that the DNA detected is from dogs in  
511 the area. We collated data across replicates and the two primer sets in an overall inventory.  
512

513 For the statistical analysis only data from species present at the southern part of the zoo was  
514 used. Detected noon-zoo animals and those also used as feed were removed from the dataset,  
515 as it was not possible to measure the exact location and biomass. The distance of the animals to  
516 the samplers was measured using a satellite view of the Copenhagen Zoo using Google earth  
517 (<https://earth.google.com/>) and using an average point of reference for animals with a large  
518 enclosure. Average body weight data was obtained from Species360 Zoological Information  
519 Management System (ZIMS) (2021). The dataset used can be found in Supplementary Table 6.  
520 We fitted a logistic model (estimated using ML) to predict detection with distance, biomass (log  
521 transformed), taxonomic group (class: bird or mammal), sampler type (WV, SF, MF) and  
522 sampling time as potential explanatory variables. Effect of sampler type, taxonomic group and  
523 sampling time were insignificant and they were removed from the model. The explanatory  
524 power of the final model (formula: detection ~ distance + log(biomass)) was substantial (Tjur's  
525  $R^2 = 0.30$ ). The model's intercept, corresponding to distance = 0 and biomass = 0, is at -1.50  
526 (95% CI [-2.29, -0.76],  $p < .001$ ). Within this model: the effect of distance is statistically  
527 significant and negative ( $\beta = -8.04e-03$ , 95% CI [-0.01, -1.68e-03],  $p = 0.015$ ; Std.  $\beta = -0.14$ ,  
528 95% CI [-0.44, 0.15]); the effect of biomass(kg) (log transformed) is statistically significant and

529 positive (beta = 0.47, 95% CI [0.34, 0.61], p < .001; Std. beta = 1.57, 95% CI [0.78, 2.52]). When  
530 not using animal biomass as a potential explanatory variable in the model, the effect of  
531 taxonomic group was significant. In this case, the explanatory power of this model (formula:  
532 value ~ Distance + Class) was weak (Tjur's R<sup>2</sup> = 0.10). The model's intercept, corresponding to  
533 distance = 0 and Class = Aves, is at -0.67 (95% CI [-1.33, -0.04], p = 0.040). Within this model,  
534 the effect of distance is statistically significant and negative (beta = -6.22e-03, 95% CI [-0.01, -  
535 1.88e-04], p = 0.046; Std. beta = -0.31, 95% CI [-0.62, -9.31e-03]). The effect of Class  
536 [Mammalia] is statistically significant and positive (beta = 1.45, 95% CI [0.81, 2.12], p < .001;  
537 Std. beta = 1.45, 95% CI [0.81, 2.12]). The data used for the logistic model can be found in  
538 Supplementary Table 6.

539

#### 540 **Authenticity**

541 Metabarcoding with universal primers that PCR amplify short fragments of the often low DNA  
542 quantities of target taxa in environmental DNA extracts comes with the inherent risk of  
543 amplifying contaminant templates <sup>e.g.</sup><sup>66</sup>. Further, library preparation, PCR and sequencing  
544 artefacts can lead to inflated diversity and false positives<sup>67</sup>. To ensure authenticity of results,  
545 we therefore followed strict sampling, laboratory and bioinformatic workflows.

546

547 To minimise risk of contamination, we created a dedicated specialised environmental DNA pre-  
548 PCR laboratory for DNA extractions of the air filtering samples in which we set up and followed  
549 guidelines commonly used for ancient DNA laboratories, such as unidirectional workflow and  
550 the use of hair net, sleeves, facemask, two layers of medical gloves, dedicated footwear,  
551 decontamination with  $\geq 3\%$  bleach<sup>57</sup>. All steps of the workflow were carried out in laminar  
552 flowhoods and using filter tips. To reduce risk of PCR introduced artefacts, we carried out only  
553 one PCR amplification for each sample PCR replicate prior to sequencing. To ensure that tag-  
554 jumps or index switching would not cause spillover in samples, we used a library preparation  
555 protocol that allowed avoidance of tag-jumps, i.e. false assignment of sequences to samples<sup>68</sup>,  
556 and twin dual-indexes to ensure that potential library bleeding would not cause false  
557 assignment of sequences to libraries<sup>69,70</sup>.

558

559 To enable identification of potential contamination, we included negative controls in all steps of  
560 the workflow and positive controls in the metabarcoding PCR amplifications. During laboratory  
561 quality control steps, we did not identify contamination in any of the negative controls. Despite  
562 this, we sequenced the negative and positive controls alongside the samples. The positive  
563 control species were not found in the sampling area, which allowed us to assess spillover  
564 between samples. We did not detect any spillover from the positive controls to the samples or  
565 negative controls, which indicated that there was no cross-contamination in the metabarcoding  
566 PCR and the following downstream analyses. No negative controls contained OTUs, except for

567 one of the negative sampling control from the water vacuum. Here, an OTU from a bird only  
568 found inside the Rainforest House (Sclater's crowned-pigeon) was detected. This OTU was  
569 further detected in the water vacuum samples collected outdoors, which took place after the  
570 collection in the Rainforest House. We detected no other OTUs from the Rainforest House in  
571 the water vacuum samples collected outside. We therefore excluded this cross-contaminating  
572 OTU from all water vacuum sampler detections. The other taxa detected with this sampler were  
573 not detected in the negative sampling controls, and were therefore determined to be true  
574 detections. No vertebrate DNA was detected in the negative controls from the 24 V and 5 V  
575 particle filters.

576

577 A crucial step to ensure authenticity was the inclusion of four PCR replicates for all samples and  
578 negative and positive controls. This was done for both markers. For each set of four PCR  
579 replicates, we used different tag combinations to lower risk of primer cross-contamination and  
580 importantly, to allow stringent filtering of sequences across each sample's PCR replicates <sup>67,71,72</sup>.  
581 We employed a conservative approach in which we only retained sequences that were found in  
582 min. three of the four PCR replicates from each sample <sup>67</sup>. Further, we only retained sequences  
583 present in a certain copy number threshold. These were crucial steps that allowed us to balance  
584 error removal with detection of diversity <sup>67</sup>. In addition, we used the LULU algorithm which  
585 removes artefactual OTUs and thereby reduces the number of false positives <sup>64</sup>.

586

587 The study was carried out in a zoo environment, which enabled us to verify the presence of all  
588 detected taxa in our collated species inventory. We are aware that we detected species known  
589 to be contaminants in laboratory reagents, such as pig, cow and chicken and to a lesser extent  
590 mouse, rabbit, goat and guinea pig <sup>73</sup>. For example, we used bovine serum albumin (BSA) in PCR  
591 amplifications which is synthesised from cow's blood <sup>74</sup>. We did however not detect any of  
592 these taxa in the negative controls. Further, cow, pig, chicken, mouse, rabbit, goat and guinea  
593 pig are present in the zoo and we therefore find our detections of them in air particle samples  
594 reliable.

595

596

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