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Duikers are infected with orthopoxviruses

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Duikers are infected with orthopoxviruses

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Abstract: An increasing number of mpox cases caused by the monkeypox virus (MPXV; *Orthopoxvirus monkeypox*), are reported in West and Central Africa, driven by frequent zoonotic spillovers into human populations that sometimes give rise to epidemic lineages[1-4]. Designing mitigation strategies to reduce spillover requires a better understanding of the ecology underlying the zoonotic emergence of MPXV[5]. Here, we show that orthopoxviruses, including MPXV, infect duikers, which are popular bushmeat in many mpox endemic regions. In bushmeat markets from the Democratic Republic of the Congo (DRC), we detected orthopoxvirus DNA in a Weyns's duiker (*Cephalophus weynsi*) and a common duiker (*Sylvicapra grimmia*) using PCR. High throughput sequencing (HTS) detected a small number of reads assigned to MPXV, and 25% of the genome of a close relative to the taterapox virus (TATV; *Orthopoxvirus taterapox*), respectively. In the Taï National Park (TNP), Côte d'Ivoire, tissue samples from a Maxwell's duiker (*Philantomba maxwellii*) carcass were also MPXV PCR positive, and 50% of the viral genome could be recovered. Suspecting scavenging as a route of exposure, we ran metabarcoding analyses on duiker faeces from TNP, which revealed that more than 21% contained non-duiker mammal DNA, including species linked to previous MPXV infections in this ecosystem. Revisiting western chimpanzee (*Pan troglodytes verus)* diet data from an mpox outbreak in TNP in 2017, supported an epidemiological link to duiker consumption[6].

Collectively, our results suggest that duikers represent a previously overlooked intermediate host for MPXV and other orthopoxviruses.

Main: Local and global mpox epidemics caused by different MPXV lineages represent a serious public health threat[7,8]. Recent evidence suggests there are frequent zoonotic spillover events of both clade I and clade II MPXV into human populations in Central and West Africa, respectively, which have the potential to start new sustained human-to-human transmission chains[1-4]. Concerningly, these spillover events appear to be increasing in frequency[9]. Understanding their source and context represents an important step towards mitigating the risk of MPXV zoonotic transmission and further spread among humans.

Multiple rodent species are hypothesized to be natural hosts for MPXV and to act as reservoirs for humans [10-12], though in Africa direct evidence for transmission from rodents to humans is lacking. In the USA, spillover to humans from prairie dogs (*Cynomys* sp.), an incidental host infected in captivity after being co-housed with African rodents, was established during an MPXV outbreak in 2003[13,14]. The best available evidence of natural MPXV transmission from a putative rodent reservoir in Africa exists for a wild non-human primate (NHP). In late 2022, consumption of an MPXV infected fire-footed rope squirrel (*Funisciurus pyrropus*) by a wild sooty mangabey (*Cercocebus atys*) caused an mpox outbreak in its social group in TNP[15].

In West and Central Africa, rodents and NHPs are frequently consumed by humans, which may create opportunities for MPXV zoonotic transmission. To assess this, between May and August 2022 we sampled wildlife meat and organs (N=666) sold on markets in the DRC, in regions where MPXV frequently emerges in humans (Supplementary Table 1, Extended Data Fig.1). Additionally, we conducted a survey of all species sold as bushmeat at the same markets. Of 744

observed animals, rodents (N=125, 16.8%), NHPs (N=216, 29.0%) and duikers (antelopes of the subfamily *Cephalophinae*; N=227, 30.5%) were the most traded (Fig. 1a, Extended Data Fig. 2). We screened all samples with a generic orthopoxvirus qPCR targeting the *P4A* gene[16]. While primate and rodent samples were negative, we surprisingly detected two PCR positive duikers: one common duiker from a market in Kikwit, a major city in Kwilu province (8.3 and 7.4 copies/µl in muscle and skin, respectively), as well as a Weyns's duiker from Kole, Sankuru province (0.7 copies/µl in muscle).

Using hybridisation capture coupled with HTS, we recovered a partial TATV-like genome from the common duiker (23.2% coverage of the reference genome at 12.3x average sequencing depth), as well as a small number of MPXV sequences from the Weyns's duiker (2.4% coverage at 2.1x depth; Table 1, Supplementary Table 2). The MPXV coverage was too low to allow for trustworthy clade assignment[17], but it seems likely this virus belonged to clade 1, as this is the only clade endemic to the DRC. While this MPXV detection could theoretically represent a contamination from an infected human, the detection of the TATV-like virus clearly shows that orthopoxviruses circulating in the wild are found in duikers on markets. These results were a first indication that bushmeat trade in Africa could be a source of exposure of humans to poxviruses, including MPXV, and that duikers may represent intermediate hosts for these viruses.

These unexpected findings prompted us to investigate a collection of tissue samples from duiker carcasses found in TNP. We focused our efforts there because of the previous evidence of MPXV endemicity in local wildlife, including a MPXV infected fire-footed rope squirrel carcass and mpox outbreaks in wild sooty mangabeys and western chimpanzees[6,15,18]. We had previously screened 44 duiker carcasses sampled in TNP between 2004 and 2017 for orthopoxviruses,

finding them all negative[6]. Here, we screened a further 72 tissue samples from 36 duiker carcasses collected between 2017-2024, spanning the period during which fire-footed rope squirrels and sooty mangabeys in TNP were infected with MPXV (November 2022 - April 2023) (Extended Data Fig.1, Supplementary Table 3). Muscle and stomach from a Maxwell's duiker carcass collected in May 2023 were positive in the orthopoxvirus qPCR (2.4 and 8.0 copies/µl respectively) as well as a specific MPXV qPCR assay (3.9 and 2.7 copies/µl respectively) (Supplementary Table 3). Only remnants of this duiker carcass were recovered, making it difficult to establish whether predation or MPXV infection itself was the probable cause of death (Supplementary Discussion). Swelling of the mesenteric lymph nodes and follicular hyperplasia in histology aligned with the clinical picture of an infectious disease, though we did not detect MPXV with PCR or immunohistochemistry in this tissue (Supplementary Figs. 1 and 2). While we were unable to culture the virus from samples of this animal, we generated a partial MPXV genome from other tissues from this carcass (52.7% coverage, 18.1x depth) (Table 1). Across the 43,585 bases which could be called applying a threshold of 3 unique reads and a majority consensus rule, this partial genome was nearly identical (99.98%) to those from the fire-footed rope squirrel and the sooty mangabeys sampled during the same year, and therefore clearly belonged to clade 2a (Fig. 2). These results are indicative of an active MPXV infection in a Maxwell's duiker with a strain closely related to those circulating more broadly in wildlife at the time. Combined with our bushmeat results, this demonstrates that wild duikers get infected with MPXV at a measurable rate in both Central and West Africa (here, 1 in 118 market samples, and 1 in 80 duiker carcasses from TNP), with viruses from clades 1 and 2 (Fig. 2).

To explore how duikers might be exposed to MPXV, we examined their diet in TNP, with the aim of identifying trophic links that could allow for transmission. Duikers are considered mostly

herbivorous, but sporadic observations and camera trap footage of scavenging on mammal carcasses suggest a carnivorous component to their diet[19,20] (Supplementary Table 4; Fig. 1b; Supplementary Information; Supplementary Videos 1–3). We applied a mammal-generic metabarcoding assay to duiker pellets collected between July 2017 and February 2020 in TNP (N=146), finding that more than 21% contained non-duiker mammal DNA (N=31; Supplementary Tables 5 and 6). Since mammal DNA can be detected from leaf swabs[21], we ran a control experiment applying the same metabarcoding approach to faeces collected from strictly herbivorous and mostly folivorous western lowland gorillas (*Gorilla gorilla gorilla*; N=46) to rule out folivory as a source of mammal DNA. Gorilla faeces yielded no non-host mammal detections, suggesting most non-duiker mammal DNA found in duiker faeces represents meat consumption. We found that duiker faeces contained the DNA of rodents (11 of 146 duiker pellets), NHPs (19 of 146), small carnivores (3 of 146), and a bat (1 of 146; Fig. 3). Notably, we detected two species, sooty mangabeys and fire-footed rope squirrels, from which MPXV-infected carcasses were found in the few months before the MPXV infected duiker carcass was sampled. These results indicate that duikers from TNP regularly scavenge other mammals that are either plausible natural or intermediate hosts of MPXV (fire-footed rope squirrels and sooty mangabeys, respectively).

To examine whether duikers might themselves act as intermediate hosts in this ecosystem, we reassessed a diet analysis of wild western chimpanzees in the lead up to a previously described MPXV outbreak in 2017, in TNP[6]. This analysis had identified the well-known heavy consumption of colobine monkeys (tribe: Colobini) by these chimpanzees (51/363 samples positive for red colobus (*Piliocolobus badius*)), but also found a single faecal sample indicating duiker consumption. Despite the fact that duiker consumption is exceedingly rare in habituated

chimpanzees in TNP (observed only 3 times over the last 46 years; Supplementary Discussion), and that the duiker-positive sample had been collected only four days before the first MPXV-positive sample (which itself originated from the same individual; Fig. 4), we had previously not considered that a duiker was a plausible source of MPXV[6]. In light of the results reported here, we now think that this MPXV outbreak in chimpanzees was likely caused by the consumption of a duiker infected with MPXV.

Although duikers have not previously been considered as potential MPXV hosts, other members of the *Orthopoxvirus* genus are well known to infect bovids, including feral vaccinia virus infecting cattle in India and Brazil[22]. Historically, bovids were also associated with the polyphyletic cowpox viruses which are occasionally transmitted to various species from their putative rodent reservoirs[23-26]. The frequent scavenging behaviour of duikers, particularly on rodents in TNP, suggests they may experience high levels of exposure to potentially zoonotic orthopoxviruses. This is supported by our detection of TATV in duiker bushmeat, a virus thus far only detected in two individuals from two African small mammal species, a Kemp's gerbil (*Gerbillus kempi*, formerly *Tatera kempi*) from Benin, and a shrew (*Crocidura* cf. *denti*) from the DRC. Scavenging may expose duikers to further zoonotic pathogens, though they have rarely been considered as sources of zoonotic diseases. One notable exception linked duikers to the emergence of the Ebola virus (EBOV; *Orthoebolavirus zairense*) during a series of EBOV outbreaks in Gabon in the early 2000s[27]. At the time, EBOV caused massive die offs in great ape populations and scavenging on their carcasses was suggested as a route of transmission to duikers. Duikers' role as intermediate hosts for EBOV was supported by the detection of EBOV RNA in a duiker bone and epidemiological data linking two human spillover events to handling of duiker carcasses. Interestingly, indigenous ecological knowledge of hunter-gatherers in

Central Africa has associated certain duiker species to carrion and disease. This has resulted in local taboos prohibiting their consumption, including for Weyns's duiker, a species in which we detected MPXV[28]. Despite this, duikers are frequently hunted and eaten by humans across Central and West Africa, where they constitute more than 60% of meat sold on bushmeat markets[29]. Duikers therefore may represent a previously underappreciated interface at which human exposure to zoonotic pathogens occurs.

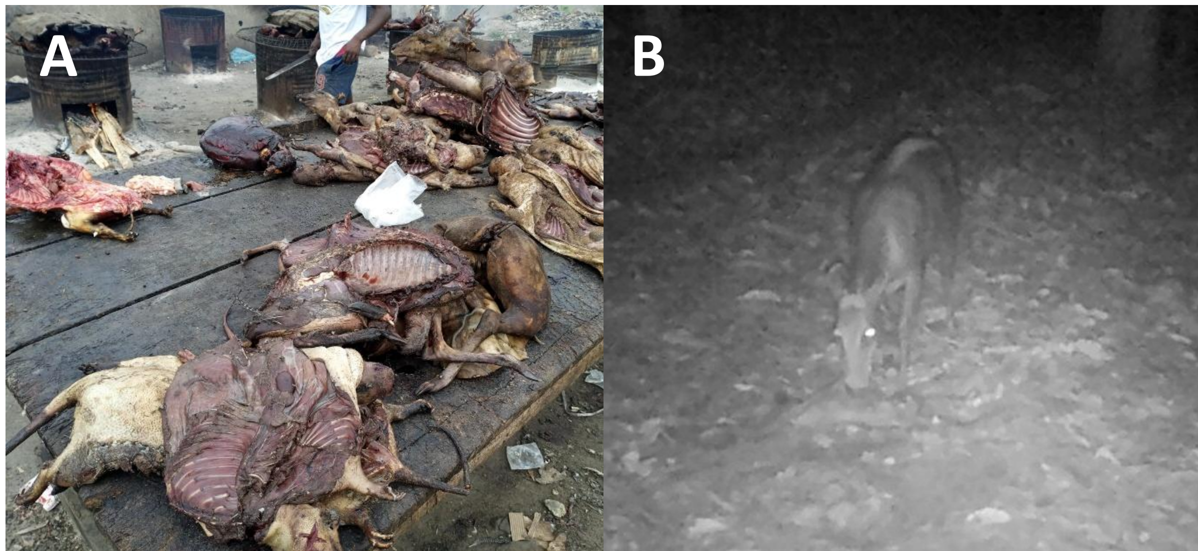


Fig. 1: A) Various wildlife species including duikers for sale at a bushmeat market in Abidjan, Côte d'Ivoire. Photo credit: Nea Yves Noma **B**) A yellow-backed duiker (*Cephalophus silvicultor*) feeding on a carcass of an agile mangabey (*Cercocebus agilis*), captured by a camera trap placed next to this carcass.

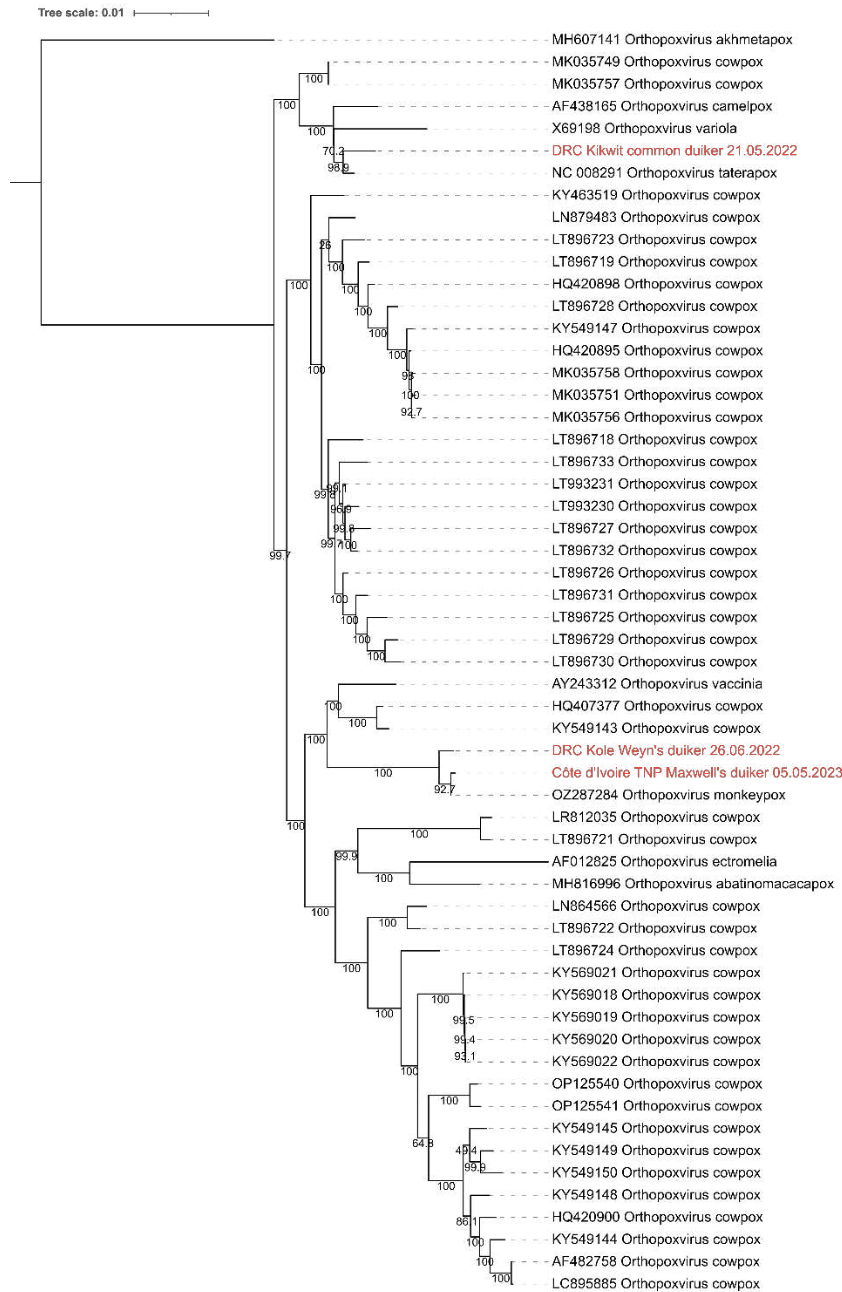


Fig. 2: Phylogeny of orthopoxviruses. This maximum-likelihood tree is derived from the analysis of an alignment comprising 87,351 nucleotide positions. Consensus sequences derived from duiker cases appear in red. We rooted the tree on *O. akhmetapox*. Branch support estimated by Shimodaira-Hasegawa-like approximate likelihood ratio tests. Scale in substitution per site.

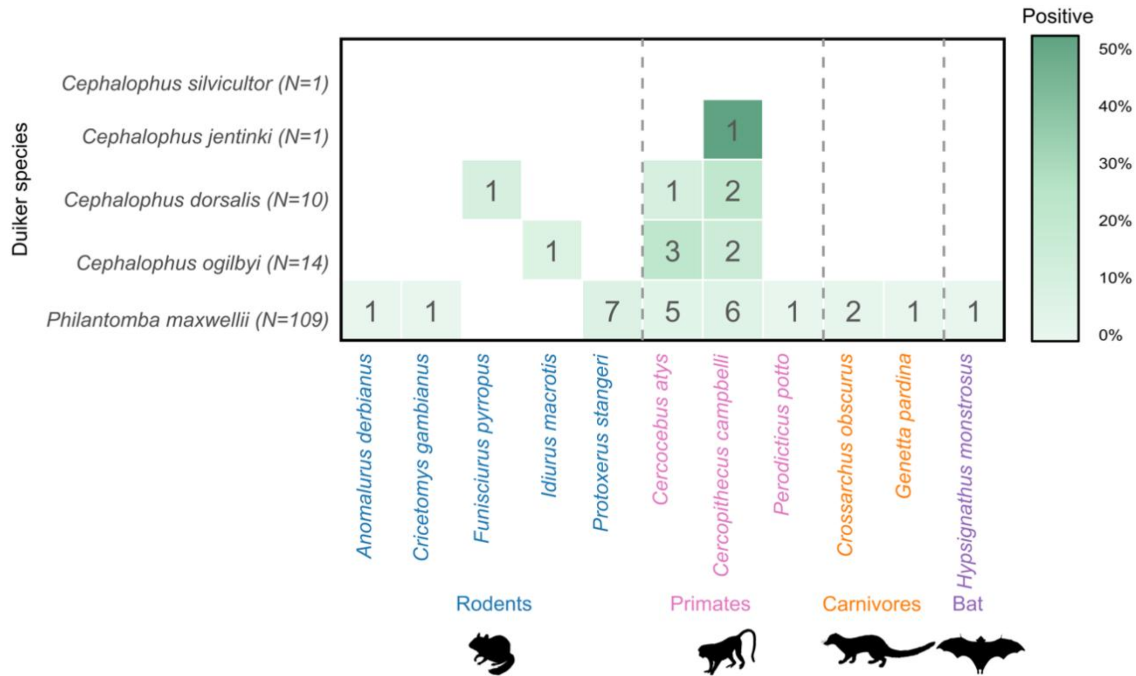


Fig. 3: Heatmap showing the proportion of faecal samples per duiker species containing DNA of different non-duiker species. Number of pellets tested per duiker species are shown on the left. Prey species are shown on the x-axis, grouped and coloured by order. Numbers given in the cells represent the number of positive pellets for a given prey species. 11 samples could not be assigned to a duiker species but did not contain non-duiker mammal detections.

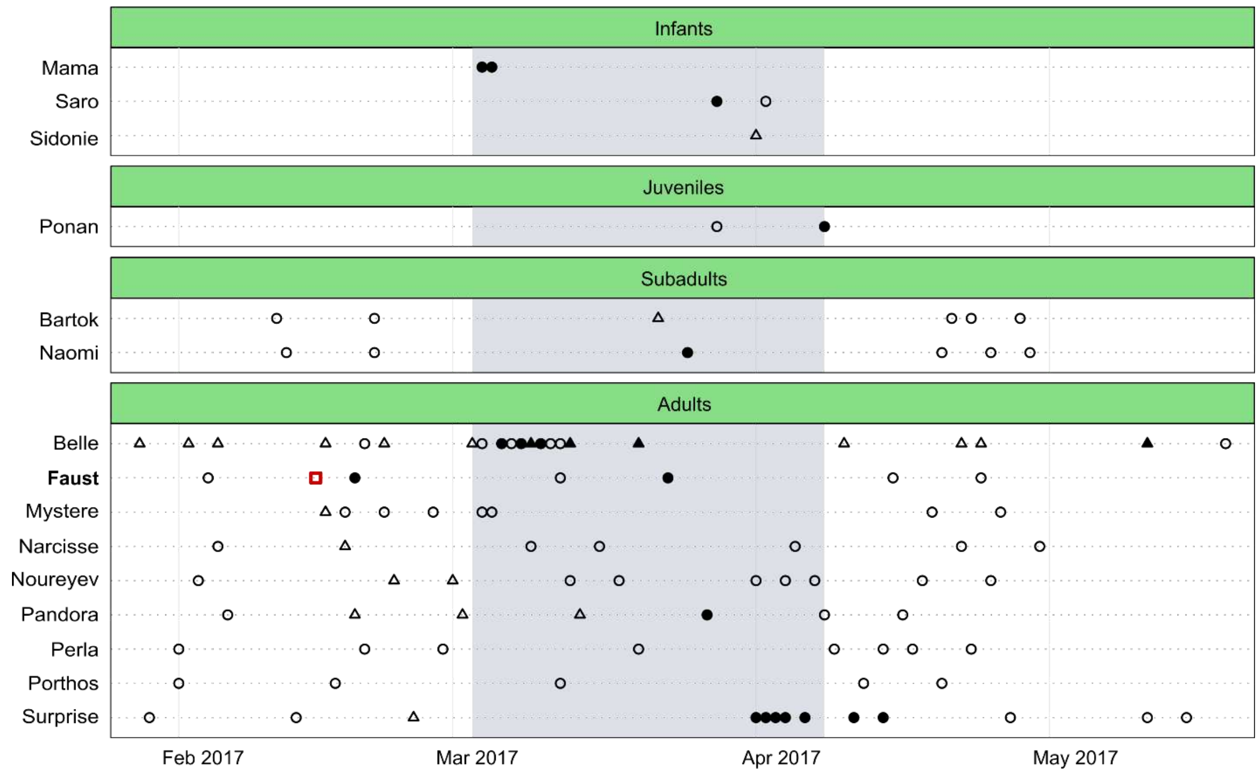


Fig. 4: **Mammal diet and MPXV detections in chimpanzee faeces** (Figure adapted from: [6]). Each line represents a different individual. Each shape represents a different sample. MPXV positive samples are represented by filled shapes (black), MPXV negative samples by empty shapes. Samples containing only chimpanzee DNA in diet metabarcoding are shown as circles. Samples additionally containing red colobus or duiker DNA are marked by triangles and red squares, respectively. The period of observed clinical signs in this chimpanzee community is shaded in grey.

Table 1. Summary statistics of best reference-based mapping results.

Sample	Depth of coverage (x)	Breadth of coverage (%)	Called bases (3x+50%)	Breadth of coverage through called bases (%)
DRC Kole Weyns's duiker	2.1	2.4	2856	1.6
DRC Kikwit common duiker	12.3	23.2	15436	7.8
Côte d'Ivoire TNP Maxwell's duiker	18.1	52.7	43585	24.0

Methods

Study sites

Democratic Republic of the Congo (DRC). MPXV is endemic in the DRC, and mpox cases have occurred in the study regions[9,30]. Bushmeat consumption is common in the country, and the trade of bushmeat is a large economic sector[31]. Wildlife is both hunted and sold locally, but is also transported much further distances before being sold[32]. Salonga National Park, Africa's largest tropical rainforest park covering 33,346 km², is an important source for bushmeat. Bushmeat samples were collected at markets, and from road-side vendors between the Salonga National Park and the capital, Kinshasa; in the predominantly rural provinces of Sankuru, Kasaï, Mai-Ndombe, and Kwilu, and the highly urbanised Kinshasa, covering ecosystems from tropical rainforest to forest-savanna mosaic (Extended Data. Fig. 1).

Taï National Park (TNP), Côte d'Ivoire. The TNP spans ~3,300 km² of primary lowland rainforest in southwestern Côte d'Ivoire and is part of the Upper Guinean biodiversity hotspot. Since 1979, the TNP houses the Taï Chimpanzee Project (TCP), a long-term primatological field site studying the ecology, behaviour and health of four habituated wild chimpanzee communities (North, South, East, and Northeast community), and a habituated sooty mangabey community[15,33]. MPXV is endemic in TNP, and mpox outbreaks have previously occurred both in the habituated chimpanzees and sooty mangabeys.

Dzanga–Sangha Protected Areas (DSPA), Central African Republic (CAR). DSPA lies in southwestern CAR forming the Sangha Trinational together with Lobéké in Cameroon, and Nouabalé-Ndoki in the Republic of the Congo. DSPA encompasses lowland evergreen and

swamp forests with bai clearings. Gorillas are habituated for tourism and research and followed daily, enabling health monitoring and rapid detection of wildlife carcasses in their territories.

Nouabalé-Ndoki National Park. Nouabalé-Ndoki National Park in the northern Republic of the Congo is part of the Sangha Trinational landscape. Here, in two long-term primatology research sites, Mondika and Goualougo, the ecology, behaviour, and health of central chimpanzees (*Pan troglodytes troglodytes*) and gorillas are studied. Both sites have been studied for more than 20 years and represent some of the best-documented populations of western lowland gorillas[34].

Sample and data collection

Bushmeat sampling and survey. Bushmeat was sold fresh or smoked and dried, from either butchered or entire animals. Tissue samples from bushmeat were collected opportunistically in agreement with the vendors using swabs, or scalpels and forceps, and wearing basic personal protective equipment (PPE), consisting of gloves, disposable plastic aprons, FFP2 masks, and safety goggles. All samples were collected in Nucleic Acid Preservation buffer (NAP) and stored at ambient temperature until they were transported to Kinshasa, and transferred to storage at -80 °C. The sampled species were identified in the field based on morphological assessment and information given by the vendors. To collect additional information on traded bushmeat where sampling was not possible, a survey recording species for sale was performed at the same markets. Vendors were selected randomly, products counted, and taxonomic groups assigned by the observing survey team.

Duiker necropsies. Wildlife mortality surveillance is conducted as part of the TCP veterinary programme, and necropsies are performed on all wildlife found dead in the research area by

specifically trained veterinarians. Following standardised field pathology and biosafety procedures, including the use of full-body PPE, a diverse range of tissue samples are collected depending on the stage of carcass decomposition[35]. Samples are collected in dry cryo tubes or in NAP buffer, and stored in liquid nitrogen until transport to a laboratory and transfer to -80°C freezers are possible. Between February 2017 and December 2024, 36 duiker necropsies were conducted by veterinary staff in the research area, the number and type of samples collected from each necropsy was dependent on the carcass decomposition state (Supplementary Table 3).

Duiker faecal sampling. Duiker faeces (N = 161) were opportunistically collected across the research area of the TCP between July 2017 and February 2020, throughout both wet and dry seasons. All faeces were visually identified as having been deposited by a duiker based on the distinct morphology of duiker pellets. Faeces were collected into 2 ml cryo tubes, which were subsequently stored in liquid nitrogen.

Camera trapping at carcasses to document scavenging behaviour. To explore the diversity of species that scavenge at carcasses, we placed camera traps in TNP and DSPA at naturally occurring vertebrate carcasses. In TNP a total of eight carcasses were monitored over a total of 51 trap nights. Scavenging was recorded at seven of these carcasses, by four different animal families, of which one was a duiker (Supplementary Table 7). In DSPA, a total of nine carcasses were monitored over a total of 20 trap nights. Scavenging was recorded at eight of these carcasses, by a total of 3 different animal families, of which two were scavenged by duikers (Supplementary Table 7).

Gorilla faecal sampling. At Mondika, faeces were collected from habituated groups Kingo, Buka, and Metetele; at Goualougo, from the Loya group. Faecal samples were collected using the clean end of a stick to scoop a pea-sized amount of faeces into a cryo tube containing NAP buffer. Samples originated from freshly deposited faeces collected directly after defecation or beneath recently vacated nests and are therefore unambiguously attributable to known gorillas, making species verification unnecessary. Samples were stored at ambient temperature until their transport to Germany, where they were transferred to -20 °C.

Laboratory analyses

DNA extraction and orthopoxvirus detection. Bushmeat samples were extracted at the animal laboratory of the Institut National de Recherche Biomedicale (INRB) in Kinshasa, DRC. The TATV-like-positive duiker sample was re-extracted at the Helmholtz Institute for One Health (HIOH) in Greifswald, Germany. For the MPXV-positive duiker sample, no tissue remained after the first extraction, precluding a re-extraction. All other sample types were extracted at the HIOH. Tissue extraction of bushmeat samples (N = 666) and duiker necropsy samples (N = 72) was performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. For the extraction of duiker pellets (N = 161) and gorilla faeces (N = 46), we used the GeneMatrix Stool DNA Purification Kit (Roboklon, Berlin, Germany) following the manufacturer´s protocol, with the following modifications. Lentil-sized faecal aliquots were transferred into bead tubes with sterile metal spoons, and homogenised using a Bead Ruptor Elite II (Biolabproducts, Hamburg, Germany) for three 20 second cycles at 4m/s. The final elution volume was 100 µL. For all sample types, each extraction batch included

a nuclease-free water control. These extraction blanks were carried through all subsequent PCR-based analyses. DNA was quantified with a Qubit 3 Fluorometer, using either the 1x dsDNA High Sensitivity (HS) or Broad Range (BR) assay kits (Thermo Fisher Scientific, Waltham, Massachusetts, USA). After extraction, work surfaces were decontaminated with 0.5 % sodium hypochlorite solution, Terralin®, and UV light for 15 minutes (min). Prior to faeces extraction, an additional decontamination step with 0.5 % sodium hypochlorite solution was carried out, due to the high sensitivity to contamination in mammal metabarcoding analyses.

Bushmeat extracts were first screened for the presence of orthopoxvirus DNA using a TaqMan Real-time PCR (qPCR) targeting the *P4A* gene at the INRB, and positive samples were confirmed at the HIOH[16]. Duiker necropsy tissues extracts were screened at the HIOH using the same qPCR assay. All extracts were tested in duplicate. PCR reactions were prepared in a total volume of 25 µl, containing 12.7 µl nuclease-free water, 2.5 µl 10x reaction buffer, 2 µl 50 mM MgCl2, 1 µl 2.5 mM dUTPs, 0.75 µl 10 µM OPV forward primer (TAATACTTCgATTgCTCATCCAgg), 0.75 µl 10 µM OPV reverse primer (ACTTCTCACAAATggATTTgAAAATC), 0.1 µl 10 µM TMgB probe (6FAM-TCCTTTACgTg+A+T+A+A+A+T+C+A+T), 0.2 µl Platinum Taq polymerase, and 5 µl of DNA template. The PCR was performed at the following cycling conditions: 10 min at 95 °C for initial denaturation, and 45 cycles of 15 seconds (s) at 95 °C and 34s at 60 °C.

The duiker necropsy extracts that were positive in the orthopoxvirus qPCR were then tested with a MPXV-specific TaqMan qPCR targeting the G2R locus[36]. PCR reactions were performed in duplicates and prepared in a total volume of 25 µl, containing 11.8 µl nuclease-free water, 2.5 µl 10x reaction buffer, 2 µl 50 mM MgCl2, 1 µl 2.5 mM dUTPs, 2 µl 10 µM G2R G forward

primer (5'-GGAAAATGTAAAGACAACGAATACAG-3'), 2 µl 10 µM G2R G reverse primer (5'-GCTATCACATAATCTGGAAGCGTA-3'), 0.5 µL of 10 µM G2R G probe (AAGCCGTAATCTATGTTGTCTATCGTGTCC), 0.2 µL of Platinum Taq polymerase and 5 µl of DNA template. The PCR was performed at the following cycling conditions: 6 min at 95 °C for initial denaturation, and 45 cycles of 5s at 95 °C and 30s at 60 °C. For the orthopoxvirus positive bushmeat samples, the MPXV-specific PCR had not been established in our laboratory, so we moved directly to library preparation, hybridisation capture, and HTS.

Hybridisation capture and high-throughput sequencing. To generate genomic data from PCR-positive duiker necropsy and bushmeat samples, Illumina-compatible dual-index libraries were generated with the NEBNext Ultra II DNA kit (New England Biolabs, Ipswich, Massachusetts, USA) following the standard protocol. Up to 750ng of DNA extract (Supplementary Tables 3 and 8) were fragmented using a Covaris ME220 Focused-ultrasonicator (Covaris, Woburn, Massachusetts, USA), set for a target fragment size of 350bp (treatment duration 45s, peak power 50, duty factor 20%, 1,000 cycles per burst, average power 10, temperature 20 °C) and used as input into library preparation. After adapter ligation, libraries were cleaned using MagSi magnetic beads (Carl Roth, Karlsruhe, Germany) with or without size selection to 300-400bp (Supplementary Tables 3 and 8). Libraries were quantified using the NEBNext Library Quant Kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA) and stored at -20 °C until further use. To enrich orthopoxvirus DNA, libraries were subjected to in-solution hybridisation capture using a custom-made kit of RNA baits (Mybaits, Daicel Arbor Biosciences, Ann Arbor, Michigan, USA) designed to cover representative *Orthopoxvirus* genomes (Supplementary Table 9)[6]. Libraries were pooled and concentrated using the MinElute PCR purification kit (Qiagen) to a final volume of 10 µl (Supplementary Table 3 and 8). We performed two rounds of 24h

hybridisation captures at 65 °C following the MYBaits Sequence Enrichment for Targeted Sequencing protocol (Version 5.0; Daicel Arbor Biosciences), with one-quarter of the recommended bait quantity. Following each round of capture, library pools were amplified to ~200ng total DNA using the Kapa Hot Start Library Amplification kit (Roche, Basel, Switzerland) and quantified using the NEBNext Library Quant Kit for Illumina (New England Biolabs). The enriched pools were diluted to recommended concentration and sequenced on an Illumina(R) MiniSeq platform using the V3 chemistry. In an attempt to increase genomic coverage, we generated multiple additional libraries from new extracts when original sample material was still available (Supplementary Tables 3 and 8).

Virus culture. Isolation of the virus was attempted for the TATV-like positive common duiker from DRC using the muscle and skin sample, as well as for the MPXV-positive Maxwell's duiker from TNP, using muscle, stomach, small and large intestine, and mesenteric lymph node samples. For the MPXV-positive Weyns's duiker, all tissue was exhausted during DNA extraction precluding virus isolation attempts. Samples were added to cell culture medium with 10% foetal bovine serum supplemented with penicillin/streptomycin (Gibco, Jenks, Oklahoma, USA) and gentamicin/amphotericin (Gibco). The tissue was homogenised on a TissueLyser II (Qiagen), and kept at 8 °C for overnight incubation. Larger particles and contaminants were removed by filtration through a 0.8µm pore membrane. Confluent layers of MA-104 cells were inoculated with the filtrate and cultivated in 12.5 cm^2 rectangular canted neck cell culture flasks, containing antibiotic-supplemented medium. Cell cultures were passaged after three days. Additionally, the bushmeat muscle sample was added to confluent layers of BHK-21 [BSR Clone 5] golden hamster kidney and HRT 18 human colon cells, processed as aforementioned and passaged after seven days. Cell lines were obtained from the Collection of Cell Lines in

Veterinary Medicine, Insel Riems, Germany. Species identity of the MA-104 cell line had previously been verified through sequencing of the cytochrome b ens-gene, species-specific PCR and PCRs targeting aldolase and RFLP. No cytopathic effect was visible for any of the samples, therefore cells were not passaged further. The lack of success in virus culture may reflect the low viral copy numbers ranging from 0 - 67.3 copies/µl depending on tissue type and animal (Supplementary Tables 3 and 8). Bushmeat samples were also stored in NAP buffer and kept at ambient temperature for several weeks, negatively impacting virus survival. Therefore, the inability to culture does not necessarily reflect the infection risk associated with these bushmeat duikers.

Histopathology. Only mesenteric lymph node samples of the MPXV-positive Maxwell's duiker were collected in 10% neutral buffered formalin, paraffin-embedded and processed for routine haematoxylin and eosin (H&E) staining. Immunohistochemistry was performed for antigen detection using the avidin-biotin-peroxidase complex method. Briefly, sections were dewaxed in xylene, followed by rehydration in descending graded alcohols. Endogenous peroxidase was quenched with 3% hydrogen peroxide in distilled water for 10 minutes at room temperature (RT). Antigen heat retrieval was performed using citrate buffer (pH 6.0) in a steamer for 20 minutes followed by a cooling period. Nonspecific antibody binding was blocked by normal goat serum (1:2 diluted in TBS) for 30 minutes at RT. The primary antibody (in-house polyclonal rat anti-cowpox serum) was applied for 1 h at RT (1:1000, diluted in TBS), the secondary biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, California, USA; 1:500 diluted in TBS) was applied overnight at 4 °C. Colour was developed by incubating the slides with ABC solution (Vectastain Elite ABC kit; Vector Laboratories), followed by exposure to 3-amino-9-ethylcarbazole substrate (AEC; Dako, Carpinteria, California, USA). The sections were

counterstained with Mayer's haematoxylin and coverslipped. As a negative control, sections were tested with pre-immune serum from rats, and MPXV infected as well as non-infected Vero76 cell pellets were included as controls. All slides were scanned using a Hamamatsu S60 scanner and evaluation was done using the NDPview.2 plus software (Version 2.8.24; Hamamatsu Photonics, Shizuoka, Japan) by a board-certified veterinary pathologist (A.B. Dipl. ECVP). Histological examination of the mildly autolytic lymph node revealed follicular hyperplasia but no evidence of poxvirus antigen (Supplementary Fig. 2).

Species identification by Sanger sequencing. An unambiguous species assignment of bushmeat and duiker carcasses based on morphology was not always possible, because bushmeat was frequently sold as cut parts and/or smoked and dried, and in TNP duiker carcasses were found with varying levels of integrity and decomposition. To molecularly verify the duiker species, we used a conventional PCR targeting a 709 bp segment of the mitochondrial cytochrome c oxidase[44], or targeting a 300bp segment of the 16S rRNA[45]. Amplicons of the expected size were either gel-purified (QIAquick Gel Extraction Kit, Qiagen; Purelink Quick Gel Extraction Kit, Thermo Fisher Scientific) or where a single clean band was visible, diluted and sent for Sanger sequencing at Eurofins Genomics (Ebersberg, Germany) or Microsynth Seqlab GmbH (Göttingen, Germany). To confirm host species identity of the duiker faecal samples, extracts were amplified using mammal-generic 16S rRNA primers (16S mam1/16S mam2 and 16S mam1/16S mam4) targeting 130 bp and 300 bp fragments, respectively[45]. Blocking primers against human and pig DNA were included to reduce amplification of potential contaminants[46,47]. Amplicons of the expected size were gel-purified (QIAquick Gel Extraction Kit, Qiagen) and Sanger sequenced by Eurofins Genomics. All resulting sequences were compared against the core nucleotide database of NCBI using BLASTn. Since this reference

database does not contain the entirety of Central African biodiversity, we cross-checked the BLASTn hits for geographic validity. If the closest BLASTn hit indicated a species which is not found in the sampling region, we checked if closely related species can be found in this region and whether sequence data for these is available. If no sequence data was available, we assigned the sample to the species which is missing from the database, but known to occur in the sampling region. If several regionally occurring species were missing in the database, we assigned the sample at genus level. For the MPXV positive duiker from DRC no extract remained to perform the species ID PCR and for several duiker necropsy samples from TNP, the PCR amplified nuclear mitochondrial DNA (NUMT) which could not be used for species assignment. In these cases, we competitively mapped HTS data to a collection of mitochondrial genomes of duikers to identify the species. For necropsy samples for which no HTS data was generated but the same NUMT was detected, we assumed they belonged to the same duiker species.

Metabarcoding of duiker faeces. To detect non-host mammal DNA, we applied a three-step 16S metabarcoding protocol designed to reduce amplification bias. A 130 bp mitochondrial 16S fragment was amplified using mammal-generic primers 16S mam1/16S mam2 together with blocking primers against human, pig, and host (cattle) DNA[48]. PCRs were run in quadruplicates with extraction blanks and no-template controls processed throughout. Amplicons of the expected size were gel-purified (Qiagen QIAquick), bead-cleaned (AMPure XP, 1.8×), dual-indexed (Nextera XT), and sequenced on an Illumina NextSeq 500 (2 × 150 bp). Negative controls were sequenced alongside samples to monitor potential cross-contamination.

Metabarcoding of gorilla faeces. Gorilla faecal DNA was analysed after the laboratory transitioned to the TagSteady library-preparation workflow to minimize tag-jumping and cross-

sample contamination[49]. Tagged mammal-generic 16S mam1/mam2 primers were used together with a human-specific blocking primer. PCRs were run in triplicate, and amplicons were pooled by plate with positive and negative controls. After end-repair, libraries were ligated to dual Illumina Y-adapters (P5–P7) and sequenced on an Illumina iSeq 100. Negative controls were sequenced alongside samples to monitor potential contamination.

Data analyses

Orthopoxvirus sequencing data analyses. Despite the generation of 6-22 libraries per infected duiker and the implementation of hybridisation capture, initial attempts at genome assembly revealed relatively low poxvirus coverage (Table 1). Therefore, we used competitive reference-based mapping on a collection of 47 complete poxvirus genomes representing all genera within the *Chordopoxvirinae* subfamily to avoid conditioning assemblies on a single reference genome. We used the BAM pipeline of PALEOMIX v1.3.10 [6], trimming reads with AdapterRemoval (--minlength: 25 –minquality: 10)[37], aligning them with BWA-MEM[38] and enabling the filtering of PCR duplicates. In all three cases, a single reference genome clearly attracted a majority of all poxvirus-like reads (70.3-96.5%; Supplementary Table 2), and a visual check of competing maps did not suggest mosaicism. Using the best reference genome maps, we then called bases applying a 3 unique reads threshold and a majority consensus rule in Geneious Prime 2024.0.3 (https://www.geneious.com). The resulting consensus sequences were used for phylogenetic analyses.

Phylogenetic analyses. To estimate the phylogenetic placement of the three consensus sequences, we first assembled a dataset comprising complete genomes from all but three orthopoxvirus species (the basal *Orthopoxvirus raccoonpox*, *O. skunkpox* and *O. volepox***)** and all publicly available genomes assigned by publishing authors to *O. cowpox* (cowpox viruses are polyphyletic and represent most of the variation within the genus)[26]. We aligned the 65 sequences (including our three partial genomes) using MAFFT v7.526[26] and identified conserved sequence blocks using Gblocks[39] as implemented in SeaView v5[40]. The resulting alignment of 87,351 positions was reduced to 57 unique sequences, and used to estimate a maximum-likelihood phylogenetic tree with IQ-TREE v3.0.1[41]. We estimated branch robustness with Shimodaira-Hasegawa-like approximate likelihood ratio tests[42], considering branches supported by values >0.90 as well supported. We plotted the phylogenetic tree using iTol v7.4.2[43].

Analysis of diet metabarcoding using Obitools. To analyse the reads resulting from these diet metabarcoding analyses, we used OBITools (v4)[50]. To construct a reference database, we downloaded the EMBL collection of mammal sequences (MAM, MUS, and ROD collections; release dated 2024/07/10) and performed an in-silico PCR using the *obipcr* command*,* allowing up to three primer mismatches and retaining amplicons between 50 and 150 bp. Resulting sequences were taxonomically annotated and filtered with *obigrep*, to retain records with valid species-, genus- and family-level assignments, with taxonomic identifiers updated against an NCBI taxdump (dated 2025/08/20). Reference sequences were deduplicated by taxonomic identifier using the *obiuniq* command and indexed with *obirefidx* to generate the reference database used for downstream taxonomic assignment.

Paired-end Illumina reads from the metabarcoding experiments were merged using *obipairing*, requiring a minimum overlap of 20 bp and a minimum sequence identity of 0.90 between read pairs. Only successfully joined read pairs were retained. Low-quality joins and poorly aligned sequences were removed, excluding reads with normalized alignment scores below 0.80. Reads were further filtered by length, retaining sequences between 80 and 2000 bp. Following read merging and quality filtering, primers were removed and for the gorilla experiments that used tagged primers, reads were assigned to their respective PCR replicate by using their 5' nucleotide tags. Sequences were subjected to dereplication using the command *obiuniq*, with sequence records annotated with read counts using the command *obiannotate*. PCR and sequencing artefacts were filtered using *obiclean*, applying a relative abundance threshold of 0.05 for variants of sequences to be retained. Taxonomic assignment was performed using *obitag* and the reference database. Assignments were categorized based on sequence similarity to reference entries, and only sequences with a best identity score ≥0.95 were retained as high-confidence taxonomic assignments. Sequences with lower identity scores or without valid matches were retained separately for downstream inspection against the core nucleotide database of NCBI using BLASTn. Final taxonomic assignment tables, including read counts and taxonomic ranks, were exported using *obicsv*.

For the duiker samples which were processed with untagged primers, we considered a detection event as genuine if it appeared in at least 10 reads and made up a minimum of 1% of the total assignable reads. One contaminated extraction control contained mammal DNA, so all co-processed samples were excluded (N=15); thus, we were able to examine the presence of non-duiker DNA in 146 duiker samples. In addition, those taxa found in this contaminated control (*Cercopithecus diana, Piliocolobus badius* and *Procolobus verus*) were not considered as

detection events in any of the other samples. For the gorilla samples, all negatives remained free of mammalian reads, and the positive control (brown long-eared bat, *Plecotus auritus*) was detected only in the positive-control wells and never in other pools. For the gorilla samples, which were processed with tagged amplicons, we would have considered a species detection event genuine when at least two of the three replicates contained at least 10 reads and made up a minimum of 0.1% of the total assignable reads in that replicate. Using these criteria we did not detect any species besides gorilla.

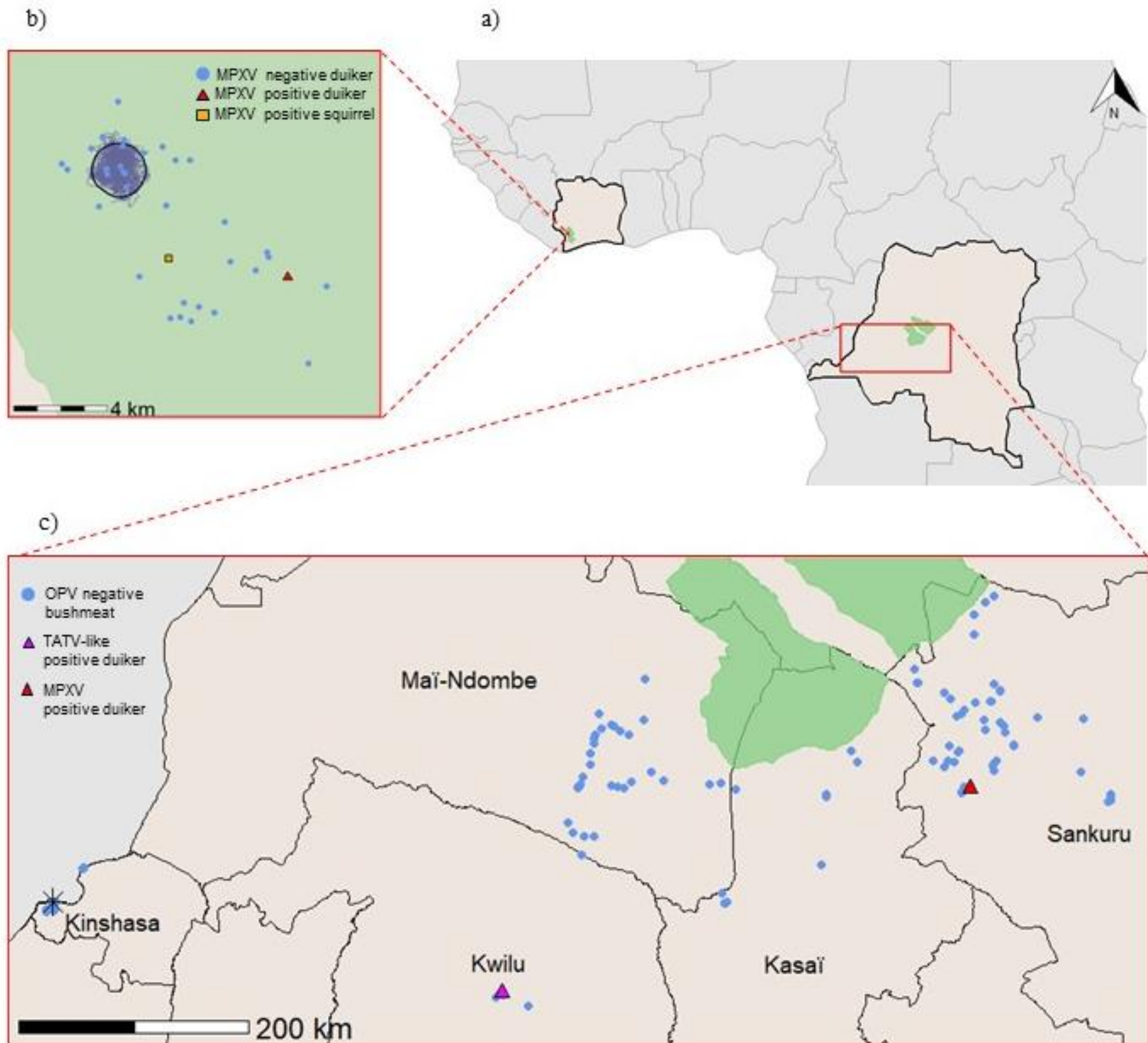
To account for potential non-independence among duiker faecal samples collected from the same individual or scavenging event, we applied a spatial–temporal downsampling procedure. Samples collected within five days and 300 m of one another were considered potentially non-independent, based on the typical faeces decomposition time under tropical conditions (≤ 5 days) and the approximate home-range diameter of 280 m for Maxwell's duikers, the most frequently sampled duiker species[20]. To remove such duplicates, we generated 100 replicate datasets, and in each replicate, one sample from every pair that met these proximity criteria was randomly omitted. This process produced independent datasets (mean = 123 number of samples, range = 122–125), and across these downsampled replicates, we saw very consistent patterns of duiker scavenging across the species (mean percentage of samples containing non-duiker mammal DNA = 20.6%; range = 19.4-22.1%; Supplementary Table 10).

Data availability

Raw reads resulting from orthopoxvirus target enrichment in the samples are available in the European Nucleotide Archive under project accession number PRJEB110094, while the consensus sequences generated from these data, as well as the sequence alignment files used in

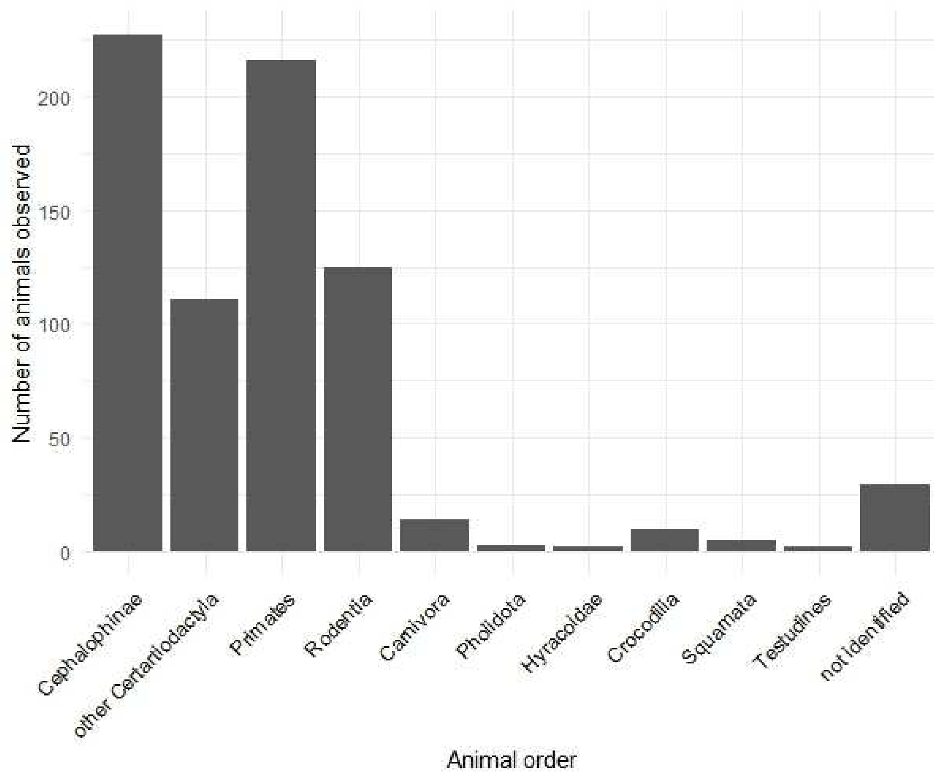
subsequent analyses are available via Zenodo (10.5281/zenodo.19068629). The results of our mitotyping efforts have been uploaded to Zenodo (necropsy samples: 10.5281/zenodo.19068543; bushmeat samples: 10.5281/zenodo.19068489). Raw reads from the duiker faeces mammal metabarcoding experiments are available via Zenodo (10.5281/zenodo.19047621) and the raw reads from the gorilla mammal metabarcoding experiments, as well as the information on the tagged primers used for each sample are also available via Zenodo (10.5281/zenodo.19046647). Consensus sequences used for duiker species identification of the faecal samples are also available via Zenodo (10.5281/zenodo.16948942; 10.5281/zenodo.17012161).

Extended Data



Extended Data Fig. 1: Study sites. A) Location of Taï National Park (TNP) in Côte d'Ivoire (left), and the bushmeat sampling area in the Democratic Republic of the Congo (DRC) shown by a red square (right), with Salonga National Park highlighted in green for reference. **(B)** Necropsy sampling locations within TNP and the home range of the habituated sooty mangabey (*Cercocebus atys*) group. The home range is approximated as the 95% minimum convex polygon

(dark purple) based on mangabey movements tracked over one year (purple). **C)** Sampling locations of bushmeat within the DRC. The site with a MPXV positive finding is marked as a red triangle, the site of a TATV-like positive finding is marked as magenta triangle, locations with orthopoxvirus-negative findings are visible as blue dots. Borders of provinces are marked as black lines, Kinshasa (black star) and Salonga National Park (green) for reference.



Extended Data Fig. 2: Total numbers of animals observed at bushmeat markets. Animals are sorted by taxonomic order, except for Cetartiodactyla which are sub-divided in duikers (Cephalophinae) and other Cetartodactyla including other Bovidae (*Tragelaphus spp*.), Suidae and Tragulidae.

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