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Pathogens spread by high-flying wind-borne mosquitoes

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Mosquito-borne diseases such as malaria and dengue threaten billions of people and cause the death of hundreds of thousands annually. Recent studies have revealed that many mosquito species regularly engage in high-altitude wind-borne migration, but its epidemiological significance remains unclear. The hypothesis that high-flying mosquitoes spread pathogens over long distances has not been directly tested. Here, we report that high-flying mosquitoes are commonly infected with arboviruses, protozoans, and helminths and provide a insights into this pathogen–vector aerial network. A total of 1,017 female mosquitoes intercepted on nets suspended from helium balloons at 120 to 290 m above ground over Mali and Ghana were screened for infection with arboviruses, Haemosporida, and filariae. The mosquitoes collected at altitude comprised 61 species, across 10 genera, dominated by *Culex*, *Aedes,* and *Anopheles*. Infection and infectiousness (capacity to transmit a pathogen to another host inferred based on disseminated infection) rates of migrant mosquitoes were 7.2% and 4.4% with *Plasmodium* spp., 1.6% and 0.6% with filariae, and 3.5% and 1.1% with flaviviruses, respectively. Twenty-one mosquito-borne pathogens were identified, including Dengue, West Nile, and M'Poko viruses, 15 avian *Plasmodium* species including *Plasmodium matutinum*, and three filariids, including *Pelecitus* sp. Confirmed head–thorax (disseminated) infections of multiple pathogens in *Culex perexiguus*, *Mansonia uniformis*, and *Anopheles squamosus* reveal that pathogens carried by high-altitude wind-borne mosquitoes are capable of infecting hosts far from their departure location. This high-altitude traffic of sylvatic pathogens (circulating in wild animals) may be key to their maintenance among enzootic foci as well as initiating outbreaks at distant locations.

mosquito-borne pathogen | dispersal | disease-spread | high-altitude windborne migration | disease surveillance

Significance

Mosquito-borne diseases threaten public health and food security. Long-range spread of these pathogens has been regarded as reflecting human or animal movement. By screening approximately one thousand mosquitoes captured on nets suspended from helium balloons at 120 to 290 m above ground, we detected high rates of infection with arboviruses, protozoans, and helminths. Twenty-one mosquito-borne pathogens of vertebrates were identified including Dengue, West Nile and M'Poko viruses, *Plasmodium matutinum*, and *Pelecitus* spp. Disseminated infections indicate that a substantial proportion of high-flying mosquitoes are likely capable of infecting hosts far from their departure location. Traffic of sylvatic pathogens (circulating among wild animals) at altitude may be key to their maintenance among disease foci and for initiating outbreaks at distant locations, as well as to disease prevention and control.

Wind-borne insect migration at altitude occurs regularly on massive scales in terms of biomass and distance that extend up to hundreds of kilometers per night (1–6). Migration, defined as a persistent movement temporarily unaffected by immediate cues for food, reproduction, or shelter, with a high probability of relocating the animal to a new environment (7, 8) fits these flights and will be used here. Insects that transmit pathogens, other pests, and species vital for ecosystem vigor are common among high-altitude flyers (1–3, 5, 6). However, migrations in tropical mosquitoes are poorly understood and questions about their effects on mosquito survival, reproduction, range expansion, spread of insecticide resistance, and epidemiology of vector-borne diseases remain enigmatic despite their potentially large impacts (9–16). The hypothesis of pathogen spread by high-altitude wind-borne mosquitoes is not new (2, 11, 17–24), but it has been supported largely by epidemiological and meteorological inferences, while direct evidence of the regularity of such movements, particularly of the infection of high-altitude wind-borne mosquitoes, has been elusive. Recent studies in Africa revealed that many mosquito species engage in wind-borne migration at altitude, i.e., 40 to 290 m above ground level (agl), on a regular basis (25–27) along with myriads of other insects (3, 28). Additional support for this hypothesis was provided by the findings that the migrants were dominated by gravid females that had fed on vertebrate blood before engaging in migratory flight, that the flights coincided with the disease-transmission season, and that many of these species have been previously implicated as vectors of pathogens (15, 25, 26). Here, we show that high-flying migrant mosquitoes of diverse taxa are often infected with arboviruses, *Plasmodium* spp., and filariae. Furthermore, migrating mosquitoes are not only infected i.e., exposed to these pathogens, but are already likely infectious, i.e., presenting a disseminated infection to the haemocoel and likely to the salivary glands, highlighting their probable capacity to transmit pathogens upon landing in a distant territory.

The authors declare no competing interest.

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Results

Aerial Mosquito Diversity. Of 1,247 mosquitoes collected at altitude (120 to 290 m agl) over West Africa during 191 collection nights between 2018 and 2020 and subjected to molecular analysis, the specific identity of 994 mosquitoes were confirmed by mitochondrial cytochrome c oxidase I *(COI)* barcode sequencing (253 were identified to subfamily; *SI Appendix*, Table S1), yielding 61 species across 10 genera (Fig. 1 and *SI Appendix*, Table S1). Diversity of *Culex* was highest (25 species), followed by *Anopheles* (11 species), *Aedes* (10 species), *Coquillettidia* (4 species), *Uranotaenia* (3 species), *Mansonia* (3 species), *Mimomyia* (2 species), *Eretmapodites* (1 species), *Aedeomyia* (1 species), and *Lutzia* (1 species). Among those identified to species, *Culex watti, Culex perexiguus,* and *Cx. cf. watti MAFP5.C5* were the dominant taxa, comprising nearly 60% of the collections, followed by nine species, including *Coquillettidia metallica*, *Culex univittatus*, *Aedes argenteopunctatus,* and *Anopheles squamosus*, which together comprised a further 20% of the collections (Fig. 1*A*). Infrequent taxa included 32 species, e.g., each represented by 0.2 to 1.2% of the specimens (2 to 12 specimens/species); followed by 17 rare species, each represented by a single specimen (singletons, Fig. 1 and *SI Appendix*, Fig. S1). Identified taxa included primary vectors of malaria (*Anopheles coluzzii, Anopheles gambiae* s.s.) and arboviruses, e.g., West Nile virus (WNV, species *Orthoflavivirus nilense,* vectored by *Cx. univittatus*), and Rift Valley fever virus (species *Phlebovirus riftense*, vectored by *Aedes mcintoshi*) (15, 29–31). Females comprised 81% of identified specimens, with no evidence for interspecies heterogeneity in the sex composition (P = 0.065, Exact Test for contingency tables, *SI Appendix*, Table S2). Overall, gravid females consisted of 43% of identified species with fractions varying between 28% (*Aedes circumluteolus*, N = 10) to 65% (*An. squamosus*, N = 17, among-species heterogeneity P = 0.001, Exact Test for contingency tables, *SI Appendix*, Table S3).

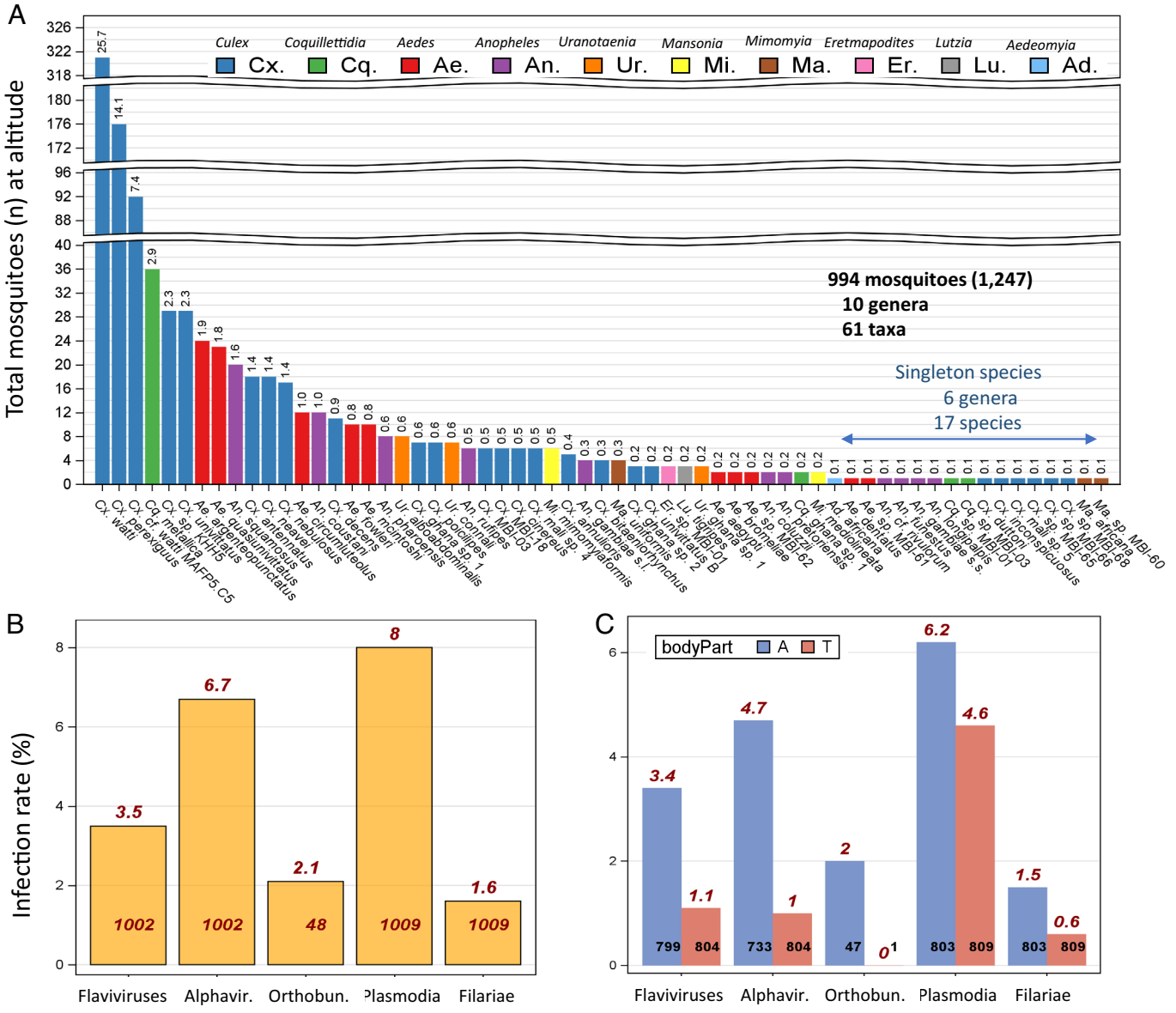

Fig. 1. Mosquito species composition at altitude and overall infection rates with major pathogen groups. (*A*) Mosquitoes identified to species are shown (N = 994, note breaks in the Y-axis; showing the number of specimens per species). The percentage of the total specimens per species is shown above bars. Species represented by a single specimen are grouped under the blue arrow. (*B*) Overall infection rates (per mosquito) of high-altitude wind-borne mosquitoes with pathogen groups assayed. (*C*) Infection rate in abdominal tissues (A, blue) and in head–thorax tissues (T, red). Infection rates expressed as percentages are shown above bars (red), and corresponding sample sizes are shown at the base.

Table 1. Overall infection rates of mosquito females intercepted at altitude (120 to 290 m above ground) with pathogens

Pathogen (lineage)	Overall[*] (N)	Abdomen (N)	Head–thorax (N)	Method[†]	Hosts[‡]	Human impact[§]
Flaviviruses	3.5% (35/1,002)	3.4% (27/799)	1.1% (9/804)	Pan-Flavi	V/A	H, L [Z,P]
West Nile Virus (1a)[¶]	0.2% (2/1,002)	0.25% (2/799)	0% (804)	Sanger	B/M	H, L [Z]
Dengue (2)[#]	0.2% (2/1,002)	0.25% (2/799)	0% (804)	Sanger	P/M	H, P [P]
Peribunyaviridae; Orthobunyavirus	2.1% (1/48)	2.1% (1/47)	0% (1)	Metagen	V/A	H, L [Z]
M'Poko virus[‖]	2.1% (1/48)	2.1% (1/47)	0% (1)	Metagen	B/M	H [Z]
Alphaviruses[**]	6.7% (67/1,002)	1.0% (8/799)	4.7% (38/804)	Pan-Alpha	V/A	H, L [Z]
Plasmodia[††]	8.0% (81/1,009)	6.2% (50/803)	4.6% (37/809)	Pan-Plasm	B/A	H [P,Z]
P. AFR006	0.1% (1/1,009)	0% (0/803)	0.1% (1/809)	Sanger	B/M	N
P. AFR146	0.2% (2/1,009)	0% (0/803)	0.3% (2/809)	Sanger	B/M	N
P. GBCAM1	0.3% (3/1,009)	0.25% (2/803)	0.1% (1/809)	Sanger	B/M	N
P. CAMBRA02	0.1% (1/1,009)	0.1% (1/803)	0% (0/809)	Sanger	B/M	N
P. TCHSEN01	0.2% (2/1,009)	0.25% (2/803)	0.0% (0/809)	Sanger	B/M	N
P. CXPER01	1.1% (12/1,009)	0.6% (5/803)	0.5% (4/809)	Sanger	B/M	N
P. matutinum (VETMED)	3.1% (31/1,009)	1.5% (12/803)	2.1% (17/809)	Sanger	B/M	N
P. relictum (SGS1)	0.1% (1/1,009)	0% (0/803)	0.1% (1/809)	Sanger	B/M	N
P. vaughani (SYAT05)	0.6% (6/1,009)	0.6% (5/803)	0.1% (1/809)	Sanger	B/M	N
P. MALNI02[‡‡]	0.1% (1/1,009)	0.1% (1/803)	0% (0/809)	Sanger	B/M	N
P. AEDMCI01[§§]	0.1% (1/1,009)	0.1% (1/803)	0% (0/809)	Sanger	B/M	N
P. CULWAT01[§§]	0.1% (1/1,009)	0.1% (1/803)	0% (0/809)	Sanger	B/M	N
P. AEDQUA01[§§]	0.1% (1/1,009)	0.1% (1/803)	0% (0/809)	Sanger	B/M	N
P. CULANN01[§§]	0.1% (1/1,009)	0.1% (1/803)	0% (0/809)	Sanger	B/M	N
P. Ghana sp. 1[§§]	0.1% (1/1,009)	0% (0/803)	0.1% (1/809)	Sanger	B/M	N
Filariae (Onchocercidae)	1.6% (16/1,009)	1.5% (12/803)	0.6% (5/809)	Pan-Filari	V/A	H, L
Cardiofilaria sp.	0.1% (1/1,009)	0.1% (1/803)	0% (0/809)	Sanger	B/M	N
Loa-like sp.	0.1% (1/1,009)	0.1% (1/803)	0% (0/809)	Sanger	U/U	N
Pelecitus sp.	0.1% (1/1,009)	0.1% (1/803)	0% (0/809)	Metagen	B,M/M	H [Z]

[*]Mosquito infection rate (regardless of bodypart, including whole-body mosquitoes) using PCR and metagenomics (positives/number tested).
[†]Pan-Flavi = Pan-Flavivirus RT-qPCR; Metagen = Metagenomics; Pan-Alpha = Pan-Alphavirus RT-qPCR; Pan-Plasmo = Pan-Plasmodium qPCR; Pan-Filari = Pan-Filariae qPCR.
[‡]V/A = vertebrate/arthropod; B/A = bird/arthropod; B/M = bird/mosquito; P/M = primate/mosquito; B,M/M = bird+mammal/mosquito; U/U = unknown/unknown.
[§]Direct effect on human (H) or livestock (L) health [P and Z denote human as primary and zoonotic host, respectively]; N = none.
[¶]One mosquito infection confirmed by Sanger (lineage 1a); other infection confirmed by WNV-specific qPCR (not sequencing or metagenomics).
[#]Infection was detected in one mosquito abdomen and another whole-body female.
[‖]Infection was detected and confirmed by metagenomics after it was detected as positive to flaviviruses.
[**]Infection with alphaviruses could not be confirmed by Sanger sequencing (N = 67) nor by NGS metagenomics (N = 48).
[††]Putative species names follow most similar sequenced isolate names in MalAvi that were clustered together by the software ASAP (*Materials and Methods*).
[‡‡]Samples with apparent mixed infection, which may result in "chimeric sequences."
[§§]New putative species based on their dissimilarity with nearest matches in the MalAvi database that were clustered separately by ASAP (*Materials and Methods*). Naming follows MalAvi convention: 3 letters of the host genus and species names followed by number (*SI Appendix*, Fig. S2).

Aerial Pathogen Diversity. Pan-genus qPCR/PCR detection assays targeting Haemosporida, filariae, flaviviruses, and alphaviruses were performed on 1,009 female mosquitoes that were captured at altitude, consisting of 803 abdomens, 809 thoraxes, and 194 whole-body specimens. Overall mosquito infection rates (infection of any mosquito part) with each pathogen group varied between 1.6% (filariae) and 8.0% (*Plasmodium* spp.; Fig. 1*B*, Table 1, and *SI Appendix*, Table S4). Infection rates for flaviviruses, *Plasmodium* spp., and filariae were higher in abdominal tissues than in head–thorax tissues (Fig. 1*B* and Table 1), probably reflecting residual pathogen DNA/RNA after exposure via bloodmeal (see also *SI Appendix*, Table S4) and early infection before pathogen dissemination beyond the midgut (abdomen) (32, 33). Sequence data confirmed pathogen infections in high-altitude mosquitoes with all pathogen groups except alphaviruses; hence, alphaviruses were excluded from subsequent analyses.

In addition to target sequencing of positive mosquitoes following qPCR/PCR pathogen detection assays, metagenomic sequences of 48 samples found positives for pan-flavivirus, pan-filaria, and pan-Haemosporida and revealed infection with viruses of other families, as well as with non-mosquito-borne pathogens (Table 1 and *SI Appendix*, Table S4). Overall, a total of 21 mosquito-borne pathogens of vertebrates were detected in this modest sample, including two flaviviruses: WNV and Dengue (DENV, species *Orthoflavivirus dengue*), one orthobunyavirus, M'Poko virus (species *Orthobunyavirus mpokoense*, MPOV), 15 avian *Plasmodium* species (*SI Appendix*, Fig. S2), and three filariid nematodes (Table 1). The *Plasmodium* spp. included the cosmopolitan *Plasmodium matutinum*, *Plasmodium relictum*, and *Plasmodium vaughani* and the African-endemic *P.* MALNI02 previously detected in Blue-billed Malimbe, *Malimbus nitens*, in Gabon (34) (Table 1). The three filarial taxa were members of different genera: *Cardiofilaria* sp., *Pelecitus* sp., as well as a taxon related to the genus *Loa* (Table 1). Natural hosts of these taxa include both birds and mammals (Table 1). These pathogens represent sylvatic (i.e., circulating among wild animals) and zoonotic (transmitted to people from an animal

natural host) species, e.g., WNV, which are transmitted by mosquitoes mainly among birds (but see DENV, Table 1). Several insect-specific viruses (viruses that infect insects, but are incapable of infecting vertebrates, e.g., Barkedji virus and Nienokue virus, *SI Appendix*, Table S4), as well as non-mosquito-borne pathogens, e.g., *Trypanosoma theileri* and *Haemoproteus coraciae* were also detected (*SI Appendix*, Table S4). Coinfection between pairs of pathogen groups (flaviviruses, *Plasmodium* spp., and filariid nematodes) did not depart from random expectations when tested in whole body, abdomens, and head–thorax body parts ($P > 0.2$, Fisher exact tests).

Pathogen–Mosquito Relationships. Infection with *Plasmodium* spp., filariae, or flaviviruses based on genus-specific PCR/qPCR was detected in 26 mosquito species intercepted at altitude, with an overall infection rate of 12.7% per female (N = 1,009), 10.7% in abdomens (N = 803), and 6.3% in head–thorax sections (N = 809, Table 1). These rates varied among mosquito species, but no systematic relationships between species' sample size and pathogen prevalence were observed (Fig. 2*A* and *SI Appendix*, Fig. S3). Both *Cx. neavei* and *Ur. connali* exhibited significantly higher overall infection rates than the means across all mosquito species ($P < 0.015$, 1 sided Monte Carlo Exact Tests for contingency tables, Fig. 2*A* and *SI Appendix*, Fig. S3). Importantly, 15 mosquito species exhibited disseminated (head–thorax) infections with these pathogen groups, a condition required for infection of salivary glands and transmission competence (Fig. 2*B* and *SI Appendix*, Fig. S4). Positive relationships between abdominal and head–thorax infection, shown by high values in both axes (Fig. 2*B*) are likely driven by susceptible, competent vectors that are more commonly exposed to these pathogens by preferably feeding on their natural host species. Such species include *Ur. connali*, *Ma. uniformis*, *Cq. metallica*, and *Ae. circumluteolus* (Fig. 2*B* and *SI Appendix*, Fig. S4). *Culex. perexiguus* is mapped close to the regression line, yet with 16% abdominal infection rate and 8% head–thorax (disseminated) infection rate, it is flagged as a vector of potential importance because of its high abundance at altitude (Figs. 1*A* and 2*B* and *SI Appendix*, Fig. S4).

Sequencing of positive mosquitoes revealed 21 mosquito-borne pathogen species (Table 1 and Fig. 2*C*). Pathogen species were often detected in several mosquito species, including in species from different genera (Fig. 2*C*). The average number of mosquito species per pathogen species was 2.1 overall (range 1 to 11), with 1.7 for (vertebrate) arboviruses, 2.1 for avian *Plasmodium*, and 1.0 for filariae. The number of pathogen species detected per mosquito species overall and as disseminated infection increased with the mosquito species sample size (*SI Appendix*, Fig. S5).

Twenty-four mosquito species infected with at least one mosquito-borne pathogen species represented seven genera, with the genus *Culex* comprising half of the species (Fig. 2*C* and *SI Appendix*, Fig. S4). Mosquito-borne pathogen richness in *Cx. perexiguus* (7) was highest, including two arboviruses (WNV, MPOV) and 5 *Plasmodium* species (Fig. 2*C* and *SI Appendix*, Fig. S5). Disseminated infections with one or more pathogens were detected in twelve mosquito species (50%, Fig. 2*C*), with *Cx. perexiguus* exhibiting the highest richness of disseminated infections (4 species; only *Plasmodium* spp. exhibited head–thorax infections; Fig. 2*C*).

Discussion

The old hypothesis that mosquito-borne pathogens are spread over large distances by wind-borne mosquitoes at altitude (2, 8, 17–19, 24, 25, 35) was based on epidemiological and meteorological inferences and on sporadic observations of mosquitoes at altitude (20, 36, 37). It was not widely accepted because it lacked direct evidence for i) the regularity and scales of such movements and

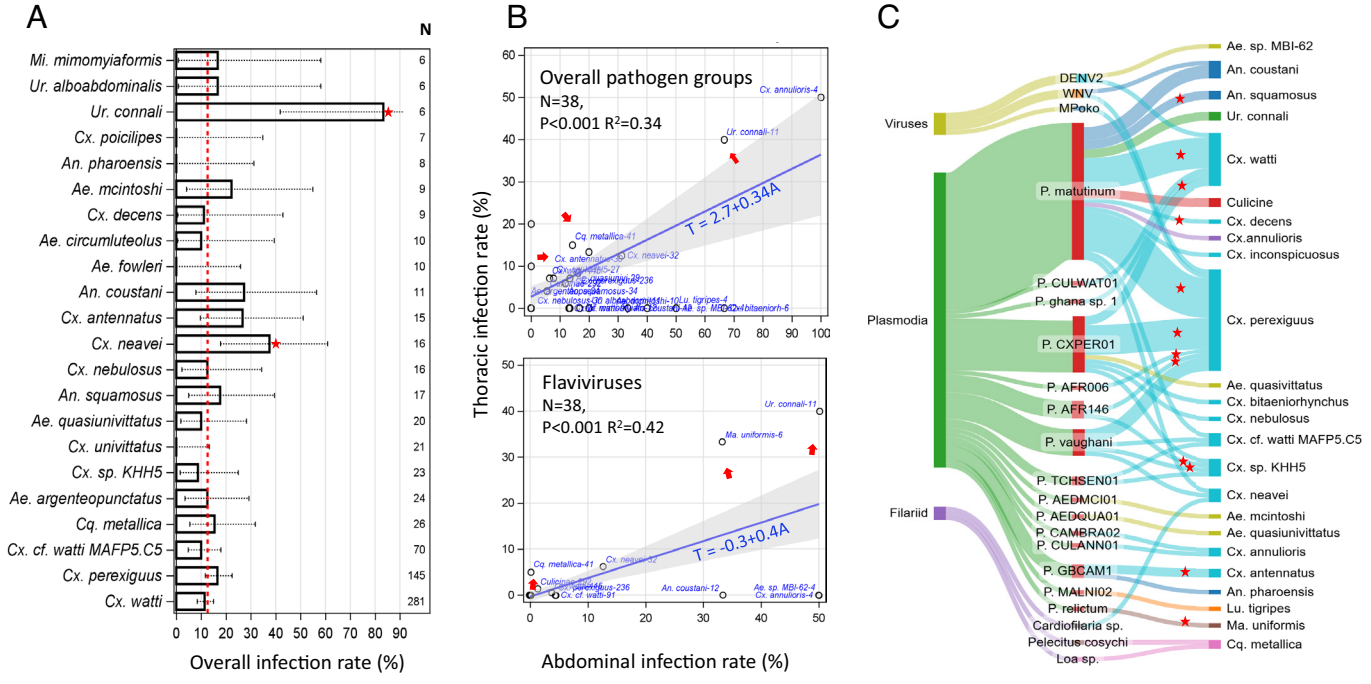

Fig. 2. Overall high-altitude mosquito infection rate per species (*A*), in head–thorax vs. abdominal tissues (*B*), and with specific vertebrate mosquito-borne pathogens (*C*). (*A*) Overall infection rates per mosquito species (N > 5, above bars) based on pan-genus PCR-based assays for flaviviruses, *Plasmodium* spp., and filariae with 90% CI. Higher infection rate ($P < 0.05$, 1-side Monte Carlo Exact Test for contingency tables) than the rate across all mosquito species (12.7%, red line) are indicated by red stars. (*B*) Relationship between disseminated (head–thorax) infection and exposure (abdominal) infection by species (abbreviated species name is followed by their sample size), with linear regression weighted by sample size (blue) and 95% CI as reference to identify outliers (red arrows). Infection with all pathogen groups (*Top*) and with flaviviruses (*Bottom*) are shown (see *SI Appendix*, Fig. S4 for *Plasmodium* and filariae). (*C*) Sankey diagram showing mosquito species infection with pathogens confirmed by sequencing. Connective band thickness is proportional to infection rate. Red stars indicate disseminated infection.

ii) infection with mosquito-borne pathogens in wind-borne mosquitoes at altitude. The notion that long-range insect migration occurs mostly in prereproductive adults (7) has also led to questioning of the role of migrants in pathogen spread. Systematic aerial collections conducted recently over Africa have revealed regular, large-scale wind-borne migration of mosquitoes (25–27), and the current results reveal high rates of mosquito infection with mosquito-borne arboviruses, *Plasmodium* species, and filariae (overall 12.7%). Importantly, high rates of disseminated infections (overall 6.3%) implicated a considerable proportion of these mosquitoes as infectious—ready to transmit pathogens when taking a blood meal after landing (16). Altogether, these results provide compelling evidence for the spread of a wide range of mosquito-borne pathogens at altitude by wind-borne mosquitoes.

Remarkably, 21 mosquito-borne pathogen species were identified from this modest sample size (1,017 female mosquitoes), including arboviruses affecting humans (dengue, West Nile, and M'Poko viruses). Infection, even with macroparasites such as filarial nematodes (Table 1), does not impede mosquitoes from undertaking high-altitude flights, confirming inferences concerning migratory blackflies infected with *Onchocerca volvulus* (38–40). Such high infection rates raise the possibility that pathogens increase migratory activity (41–43). These 21 pathogen species were detected in 24 of 61 mosquito species collected at altitude (39%). Half of these (12 species, 20% of all species) demonstrated disseminated infections (i.e., in head–thorax). Although salivary gland infection barriers can render mosquitoes with disseminated infections unable to transmit, these barriers are rare (44). Moreover, postdissemination barriers to arboviruses reduce the fraction of infectious mosquitoes, yet species that exhibited disseminated infection typically transmitted the virus to a new host (45, 46). These results reveal that wind-borne migration of diverse pathogens is widespread among multiple mosquito vector species, and lays to rest the notion this is a rare event involving one or a few super-spreader mosquitoes (Table 1 and Fig. 2).

The public health and economical importance of wind-borne pathogen spread by mosquitoes depends on the pathogen species composition, which in turn depends on the mosquito species composition, their abundance, and age (past exposure) at altitude. Additionally, it depends on mosquito displacement, which is determined by the wind speed, trajectory, and flight duration, the number of high-altitude journeys (nights) an individual mosquito takes, the selectivity of wind direction, and whether these mosquitoes survive their journey and refeed following their descent. The suitability of the landing destinations for the mosquitoes and the abundance of susceptible hosts there are also important.

The absence of human *P. falciparum* and *P. ovale*, common in people [65% in Mali (47), 73% in Ghana (48)] suggests that the majority of these mosquitoes have fed on animals rather than people. Further, at least 20 of the 21 mosquito-borne pathogen species detected circulate among wild animals and are considered sylvatic, e.g., dengue virus serotype 2 also circulates among nonhuman primates and possibly birds in West Africa (49). This highlights the value of aerial collections in surveillance of sylvatic pathogens that are especially difficult to monitor; many even lack diagnostic assays (15, 50, 51), and providing information on pathogen and vector movement trajectories, putative sources, and destinations (3, 26). Using long-flying drones or towers requires a larger initial investment but will increase collection throughput and may prove cost-effective. Pathogen spread by wind-borne mosquitoes likely connects sylvatic foci and may prevent regional extinction despite fluctuations including loss in some foci (52–55), thus could be playing a role in maintenance of these pathogens. Where these pathogens arrive at the same destinations using migratory birds (56), or human transport (57, 58), the importance of wind-borne mosquito spread diminishes. Nonetheless, wind-borne mosquitoes likely arrive in certain destinations that migratory birds avoid because they are situated away from their flyways (56) or because the bird's journey is often too long compared with the short period of viremia (3 to 6 d), and growing evidence that virus recrudescence is unlikely in birds, thus limiting the chance that an arbovirus will be carried from an origin to distant destination (59). Spring migration northward occurs when density of local vectors is low, diminishing transmission between hosts along the migration route; an obstacle that is circumvented if infected vectors arrive. In contrast to dogma, the evidence linking birds to the spread of arboviruses over medium and large distances is tenuous reflecting our inability to determine whether an infectious bird is a migrant, where it was infected, and will it continue to move (59). Finally, the massive numbers of insect migrants compared with vertebrate migrants (1, 3, 25, 60, 61) (and see below) may outweigh the transmission risk linked to wind-borne vectors even if their rates of infection are an order of magnitude lower than recorded here.

Nineteen of the 21 mosquito-borne pathogens detected in our sample circulate in birds (Table 1). This may reflect the predominance of the genera *Culex* (77% specimens, 40% species, Fig. 1*A* and *SI Appendix*, Table S1) and *Coquillettidia* (4% specimens, 6.5% species, Fig. 1*A* and *SI Appendix*, Table S1) of which many species feed on birds (62). Information on host preference for most species sampled here is scarce, and the authors could not find records on *Cx. watti*, the most sampled species or on *Ur. connali*, which showed the highest rate of infection. While most *Uranotaenia* species examined to date feed primarily upon the blood of amphibians (62), *Ur. mashonaensis* in northern Nigeria bites humans (29%); other mammals (20%), and birds (24%) (63) and *Ur. alboabdominalis* was reported biting man in Uganda (64), suggesting that exposure of *Ur. connali* to mammalian and avian pathogens is plausible. Although prevalence was not related to species sample size (Fig. 2 and *SI Appendix*, Fig. S3), the number of pathogen species detected per mosquito species increased with sample size (*SI Appendix*, Fig. S5), accounting for the predominance of avian pathogens. Sampling larger numbers of species that typically feed on mammals, such as *Aedes* and *Anopheles* (62) will likely increase the representation of mammalian pathogens. Birds can exhibit persistent infections with *Plasmodium* spp. (even over years) and exceptionally high prevalence (>90%) (65).

The bionomics and vector status of *Cx. watti*, the dominant species in our aerial collection, are unknown. It was reported infected with Sindbis virus (66), a zoonotic avian virus. We cannot rule out that *Cx. watti* transmits dengue among various hosts (49), but the detection of DENV in one specimen here is likely due to residual viral RNA from the blood meal of an infected host. *Culex perexiguus* (the 2nd most abundant species) exhibited the highest pathogen richness and the highest number of pathogens with disseminated infections (*SI Appendix*, Fig. S5). Likewise, *Cx. perexiguus* showed relatively high rates of infection and transmission potential (Fig. 2 and *SI Appendix*, Figs. S2 and S4). *Culex perexiguus*' roles in the transmission of WNV, Usutu virus (*Orthoflavivirus usutu*), Bagaza virus (*Orthoflavivirus bagazaense*), Sindbis virus (*Alphavirus sindbis*), and avian *Plasmodium* spp. in Africa, Europe, and the Mediterranean are amply demonstrated (51, 67). Its large geographical range across Africa, southern Europe, and Asia (15, 68) may well be related to its extensive migratory behavior (Fig. 1 and see ref. 26); and both traits may explain its high infection rate. Because the community of wind-borne mosquito species (>60, Fig. 1*A*) feed on a diverse set of vertebrate species (62, 69), they are likely to spread diverse mosquito-borne pathogens (15, 29, 50). The sizable fraction of pathogens that could be identified only to

genus level, without a close match in available databases (*SI Appendix*, Fig. S2 and Tables S1 and S4) suggests that the actual aerial pathogen (and mosquito) diversity is considerably larger.

Estimates of the species' aerial densities reveal that a panel density (*Materials and Methods*) of a single mosquito throughout our study (191 collection nights), is equivalent to an aerial density of 1 female mosquito/390 million m^3 of air. Because nocturnal migration of wind-borne insects at altitude advances in broad fronts, at least tens of kilometers wide (1, 28, 70), the corresponding number of mosquitoes expected to cross a sector of 100 km line perpendicular to the wind direction between 100 and 300 m agl is 11,635 per night. Accordingly, during a migration season of 4 mo, >1 million mosquitoes of each of the 17 rarest species fly at altitude over that sector alone, while the corresponding values are 10 to 200 million for each of the 12 most common species. Using a conservative disseminated infection rate of 0.1% (0.1 to 4.6%, Table 1) implies thousands to millions of potentially infectious high-flying mosquitoes per species crossing each 100 km sector. In tropical regions, mosquitoes are likely to survive a night flight at 100 to 300 m where temperature (mean = 27 °C; range = 24 to 29 °C) and RH (mean = 65%; range = 50 to 81%) are favorable (3) and predators, e.g., birds, bats, and spiders are uncommon (71). Survival in mosquitoes subjected to high altitude conditions was high (92% for 6 h). Subsequently, they laid eggs and took a bloodmeal on par with controls (16, 27), confirming 97% survival of insects collected at altitudes in nonsticky nets, which were able to feed and reproduce (72). Mosquitoes can cover tens to hundreds of kilometers per night (25). Such massive pathogen pressures may greatly exceed the numbers of infectious terrestrial hosts that arrive in the same areas and thus may be the ultimate driver of mosquito-borne pathogen spread from sylvatic foci or endemic areas. Sequential spread or extreme events may extend the migrations into subtropical, Mediterranean, and even low latitude temperate zones, where susceptible populations are concentrated. Collectively, these features position wind-borne mosquito migration as an important, yet underappreciated mechanism for pathogen redistribution, bridging fragmented foci and ecosystems, sustaining transmission cycles, and reshaping the landscape of vector-borne disease risk. Public health decisions about risk of mid- and long-range mosquito-borne disease spread may consider susceptible populations downwind from active sources located up to hundreds of kilometers away and consider strategies to minimize emigration from active mosquito-borne disease transmission sources, monitoring along predominant wind "corridors," and readying rapid-response to abate local spread following indications of disease transmission in new destinations.

Materials and Methods

Study Sites. Aerial collection stations were established in Mali and Ghana: the Sahelian village, Thierola (13.659, −7.215, Mali), the Sudano-savanna village, Kenieroba (12.112, −8.332, Mali), the Guinea-savanna village, Bia (10.492, −5.910, Mali), the Guinea woodland ecozone near the town of Wenchi (7.781, −2.162, Ghana), and a moist-semi-deciduous forest near the town of Agogo (6.961, −0.960, Ghana). Locations for aerial sampling were selected rather arbitrarily based on position at a flat or rolling landscape, away from high topographic features, >10 km away from airports, and having a clearing ~500 m away from human houses, power lines, roads, and tall trees (3, 25, 27). No information on mosquito density or pathogen transmission was considered. Malian ecozones receive annual precipitation of 500 to 900 mm during the short-wet season (June–October), whereas in Ghana, annual precipitation varies between 1,200 and 1,500 mm and spreads during most months of the year (73). These study areas have been described previously (25, 58, 74), as have the field methods used in this study (3, 16, 25). Collections were made between March 2018 and October 2022. Due to logistical reasons, sampling intensity differed between localities with 50, 16, 67, 26, and 32 sampling nights in Thierola, Kenieroba, Bia, Wenchi, and Agogo, respectively.

Aerial Collection. The methods have been described in detail previously (3, 16, 25). Briefly, insect sampling was conducted using sticky nets (1 × 3 m panels) attached to the tethering line of helium-filled balloon (3 or 3.3 m diameter), with each balloon typically carrying three panels, suspended at 120, 160, and190 m agl on the 3 m balloon and 120, 190, 240, and 290 m agl on the 3.3 m balloon. Balloons were launched approximately 1 h before sunset and retrieved 1 h after sunrise, the following morning. To control for insects trapped near the ground as the panels were raised and lowered, comparable control panels were raised up to 100 m agl and immediately retrieved during each balloon launch and retrieval. Following panel retrieval, inspection for mosquitoes was typically carried out immediately, and specimens removed from the nets with forceps were briefly washed in chloroform to remove the insect-glue and individually stored in RNAlater™ (Invitrogen, Thermo Fisher Scientific, US). After several days in room temperatures in field conditions, specimens were placed in −20 °C freezers. Other insects were stored in vials containing 80% ethanol.

Specimen Processing and DNA/RNA Extraction. In the laboratory, mosquitoes were thawed on ice, placed momentarily on filter paper to absorb excess RNAlater™ solution, washed in deionized water and examined under dissecting microscope. Specimens were identified to genus or subfamily, their sex and gonotrophic state observed and recorded according to Sella scores: 1–2 for bloodfed, 3–5 for semigravid, and 6–7 for gravid (75). Initially, DNA/RNA extraction was carried on whole specimens. Later, the female's abdomen was dissected from her thorax, and each part was independently subjected to nucleic acid extraction as were whole bodies of male mosquitoes. Extractions following standard protocols are described in *SI Appendix*, Supplementary Results and Discussion. To preserve both RNA and DNA, no DNAses were added at the last steps of the extraction.

Mosquito Identification and Pathogen Screening. The mosquito mitochondrial *cytochrome oxidase I* (*COI*) gene was PCR amplified (76) using barcoded primers (identifying each amplicon) using 1.5 μL of the nucleic acids extracts (above). The 658-bp amplicons were sequenced using the Oxford Nanopore Technologies long-read MinION NGS platform, following established protocols (77, 78). A small minority of the specimens were subjected to standard PCR followed by Sanger sequencing (Eurofins Genomics, USA) of their amplicons after purification using the QIAquick PCR Purification Kit (QIAgen, USA).

Samples consisting of RNA/DNA extractions of females were screened individually for pathogen groups including flaviviruses, alphaviruses, Haemosporida, and filariae using group-specific real-time PCR assays with primers (and associated probes, *SI Appendix*, Table S5). To detect infection with Haemosporida, mosquitoes were screened with Haemosporida qPCR targeting the *COI* following an established protocol (79). Positive samples, defined as having CT<36 (80) were subjected to nested PCR amplification targeting 477 bp and 799 bp of *cytochrome b* (*cyt b*) (81–83) (*SI Appendix*, Table S5). *Anopheles* mosquitoes were also screened for human *Plasmodium* spp. using the qPCR assays of Bass et al. (84).

To detect infection with filarial nematodes, mosquito samples were screened using qPCR targeting *28S rRNA gene* (85). Positive samples subjected to standard PCR of the *COI gene* were confirmed by visualizing amplicons on 2% agarose gel (85) and sequenced using Sanger sequencing (Eurofins Genomic, USA). Infection with arboviruses of the genera Orthoflavivirus (and Alphavirus) were screened in the abdomens and head–thorax portions of mosquitoes by RT-qPCR associated with a high-resolution melting curve (86) using one step mixes (GoTaq® 1-Step RT-qPCR System, Promega, US). Positive flaviviruses samples were subjected to a nested PCR on a 960 bp fragment of the NsP5 gene (87), and purified amplicons were sequenced by Sanger sequencing (Eurofins Genomics, MD US). A subset of 48 samples were sequenced using the ONT MinION platform (below).

All PCR and qPCR assays had at least one negative (molecular grade water) and one positive control per assay. *Plasmodium falciparum*, *Brugia malayi*/*Dirofilaria immitis*, dengue virus, and Eilat virus (*Alphavirus Eilat*) were used as positive controls for the pan- Haemosporida, pan-nematode, pan-flavivirus, and pan-alphavirus assays, respectively. Most positive specimens by qPCR/PCR were amplified and sequenced twice, often with alternate primers. Verifications were

carried out in spaced wells. Repeated PCR and sequencing of the mtCOI using thorax-head and abdomen showed high agreement between sequences. None of the wild positives detected *P. falciparum*, *D. immitis*, or another pathogen present in our laboratory, corroborating that contamination was well controlled, except dengue, in which genetic divergence between the DNEV control and the field isolates was high (total of 53 substitutions in 952 bp fragment).

Metagenomics on Selected Positive Samples. Metagenomic analyses on 48 individual mosquito abdomens found to be positive for flaviviruses (N = 29) or *Plasmodium* spp. (N = 10) or filariae (N = 9) divided into two pools of 24 samples were carried out using the MinION nanopore platform in two separate flow cells following methods as described in Supplementary Text.

Bioinformatics and Phylogenetic Analysis. The mosquito *COI* amplicon sequences, generated using the MinION were analyzed through ONTbarcoder version 1.9 or 2.0. Sequences of individual mosquitoes (amplicon metagenomics consensus per individual) and pathogens obtained by Sanger sequencing were blasted against repositories in BOLD, GenBank (NCBI) and, unless otherwise specified, species identity was provisionally given if sequence similarity was ≥98% (in most cases, it was ≥99%). Neighbor-joining (NJ) phylogenetic trees were used to cluster the sequences derived here in comparison to best matches from corresponding databases. Mosquito taxa that did not fit these criteria were provisionally named (e.g., *Aedes mali* sp. 1, *Culex* MBI-61) based on their sequence (or subfamily), in cases where molecular amplification failed repeatedly.

Metagenomic sequence database-calling and demultiplexing was accomplished on the device with the MinKNOW operating software v21.11.7 (Oxford Nanopore Technologies). See Supplementary Text for details.

Phylogenetic relationships among *Plasmodium* partial *cytb* gene sequences (470 bp) obtained in this study and assignment into putative species was done by mapping clusters generated by the software Assemble Species by Automatic Partitioning (ASAP) (88) on a phylogenetic tree that combined these isolates with previously reported haemosporidian sequences as described in Supplementary Text.

Species-specific mosquito exposure to blood was estimated as the fraction of gravid, semigravid, and blood-fed females combined as opposed to unfed females. Species-specific and whole sample infection rates were estimated across mosquito specimens with data on a particular pathogen (e.g., DENV) and groups of pathogens (flaviviruses, or vertebrate pathogens). Overall mosquito infection rate was estimated based on detection of either flavivirus, *Plasmodium* spp., or nematodes in any part of a mosquito (data on alphaviruses were not used because sequencing failed to confirm infection detected by the qPCR assay). In calculating infection rates, super-infected mosquitoes (infected with >1 pathogens, or in both body parts), were considered as single positives for that pathogen or pathogen groups. Disseminated infections were calculated based on the thorax and head body part alone. Abdominal infection rates were estimated based on that body part alone.

Statistical Analysis. Heterogeneity among species was evaluated using the contingency table likelihood ratio chi-square test after pooling species with sample size <6. If the fraction of cells with expected counts <5 was greater than 20%, we used exact tests based on Monte Carlo simulation of 10,000 samples using Proc Freq (89). Weighted regression analysis (using sample size per species as weight) and local regression to assess trends between variables were calculated using Proc Reg and Proc Sgplot in SAS (89).

Aerial density was estimated using the panel density of the species divided by the total air volume that passed through that net that night (i.e., aerial density = panel density/volume of air sampled, and volume of air sampled = panel surface area × mean nightly wind speed × sampling duration). The panel surface area was 3 m^2. Wind-speed data were obtained from the atmospheric reanalyses of the global climate (ERA5). Hourly data consisting of the eastward and northward components (horizontal vectors) of the wind were available at 31-km surface resolution at 2 and 300 m agl (1,000 and 975 mbar pressure levels). Overnight records (19:00 through to 06:00) were averaged to calculate the nightly mean direction and mean wind speed over each African sampling station based on standard formulae using code written in Base SAS (89). Nightly collections reflecting duration panels were suspended at altitude from 18:00 to 08:00 the following day (14 h sampling duration/night). The intensity of migration was expressed as the expected number of migrants/species crossing a line of 1 km perpendicular to the wind direction at altitude, which reflects their direction of movement (1, 3, 28, 90). We used the mean wind speed at altitude during the migration season (4.5 m/s) and assumed that the mosquitoes fly in a layer depth of 200 m agl (3, 25). The nightly migration intensity was computed across the 4-mo flight season (in which most species were sampled), including sampling nights during which no migrants were captured). The corresponding annual index was estimated for a sector of 100 km following previous publications (3, 25).

Data, Materials, and Software Availability. All data used in this paper are publicly available as follows. Mosquito species identification, sexual and gonotrophic state composition, and infection status: *SI Appendix*, and as a dataset in repository Figshare (91). The mosquito species-diagnostic mtDNA COI sequences are also publicly available in the Figshare repository (91). Metadata and sequence data of mosquito-borne pathogens (and other microorganisms detected) are available in the Figshare repository (92).

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