Anopheles stephensi Mosquitoes as Vectors of *Plasmodium vivax* and *P. falciparum*, Horn of Africa, 2019

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Anopheles stephensi mosquitoes, efficient vectors in parts of Asia and Africa, were found in 75.3% of water sources surveyed and contributed to 80.9% of wild-caught *Anopheles* mosquitoes in Awash Sebat Kilo, Ethiopia. High susceptibility of these mosquitoes to *Plasmodium falciparum* and *vivax* infection presents a challenge for malaria control in the Horn of Africa.

Malaria control programs in Africa traditionally focus on rural settings, although transmission is also a health concern in some urban settings (*1*). *Anopheles stephensi* mosquitoes breed predominantly in urban settings, prefer water storage containers (*2*), and are found throughout the Horn of Africa (*3*). To determine susceptibility of *An. stephensi* mosquito vectors to infection with local *Plasmodium* strains, we measured their abundance in an urban area of Ethiopia and characterized their aquatic habitats, biting and resting behavior, and competence to transmit local *P. vivax* and *P. falciparum*.

Study protocol was approved by the Institutional Ethical Review Board of the Aklilu Lemma Institute of Pathobiology of Addis Ababa University (ALIPB IRB/025/2011/2019), the Oromia Regional Health Bureau (BEFO/MBTFH/1331), and AHRI/ALERT Ethics Review Committee (AF-10-015.1, PO07/19). All participants or parents/legal guardians for participants <18 years of age provided written informed consent. Persons who volunteered for human landing collection also provided written informed consent, were monitored for 3 weeks after collections, and if symptomatic and positive received treatment for *Plasmodium* according to the treatment guidelines of the country.

The Study

This study was conducted in Awash Sebat Kilo, Ethiopia, an area of perennial malaria transmission, during April–September 2019. We examined aquatic habitats for immature-stage *Anopheles* mosquitoes by standard dipping (10×/site) for 5 consecutive days (*4*). We assessed mosquito resting, feeding, and host-seeking behavior by 5 methods: CDC miniature light traps model 512 (John W. Hock Company,

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https://www.johnwhock.com), human landing collection, pyrethrum spray sheet collection, aspiration from animal shelters, and cattle-baited traps (*5*). We identified adult mosquitoes by using standard keys and confirmed identification by targeted sequencing of nuclear internal transcribed spacer 2 (ITS2) and mitochondrial cytochrome oxidase subunit 1 gene (COI) (*6*). To generate clade topologies, we compared *An. stephensi* mosquito DNA sequences with those in publicly available libraries (*7*). We determined mosquito blood meal sources by using multiplex PCR targeting cytochrome b (*8*) and infection status by using 18S rRNA nested PCR (*9*).

Adult *An. stephensi* mosquitoes reared from immature mosquitoes from local water sources and a colony of *An. arabiensis* mosquitoes (≈120 each) were fed in the dark for 30 min on membrane feeders containing fresh blood from Adama malaria clinic patients with microscopy-confirmed mono- and mixed-species infections with *P. vivax* and *P. falciparum* (*10*). Unfed and partially fed mosquitoes were removed; fully engorged mosquitoes were maintained on sugar solution. At 7 or 12 days after feeding, mosquitoes were dissected, their midguts were examined for oocysts, and their salivary glands were examined for sporozoites. To compare infection status between *An. arabiensis* and *An. stephensi* mosquitoes, we performed logistic regression. We used individual mosquito data and a fixed effect for each patient to account for correlations between mosquito observations from the same donor. Bland-Altmann plots were generated for differences in infectivity between mosquito sources by using the Pitman test of difference in variance. For analyses, we used STATA version 13 (StataCorp., https://www.stata.com/company) and GraphPad Prism 5.3 (GraphPad Software Inc., https://www.graphpad.com). Raw data have been deposited in the DRYAD data depository (https://datadryad.org/stash/dataset/doi:10.5061/dryad.gf1vhhmnt).

An. stephensi larvae were detected in 75.3% (64/85) of the 85 artificial water sources surveyed (Table 1). A total of 49,393 immature *Anopheles* larvae and pupae were collected during 20 weekly collections in April–September 2019, of which 45,316 (91.7%) emerged as adult mosquitoes in the laboratory. Morphologic identification of adults confirmed that all were *An. stephensi*. During monthly rounds of entomologic surveillance in August and September (6 days each), we collected 89 adult female *Anopheles* mosquitoes (72 [80.9%] *An. stephensi*, 16 *An. gambiae*, and 1 *An. pharoensis*). We detected *P. vivax* in 2.8% (2/72) and *P. falciparum* in 1.4% (1/72) of wild-caught *An. stephensi* mosquitoes. Blood meal source was identified for 35.0% (28/80) blood-fed wild-caught *An. stephensi* mosquitoes; exclusive human blood meal was identified for 17.2% (5/29). The remainder fed (multiple blood meals) either on humans and animals (n = 9) or animals only (n = 14) such as goats (n = 21), cows (n = 4), and dogs (n = 5). Successful sequencing of ITS2 for 76 and COI for 45 *Anopheles* mosquitoes confirmed that all were *An. stephensi*. According to ITS2 sequences, *An. stephensi* mosquitoes from Ethiopia formed a well-supported monophyletic clade with isolates from the Arabian Peninsula and Southeast

Table 1. Characteristics of 85 aquatic habitats surveyed in study of *Anopheles stephensi* mosquitoes as vectors of *Plasmodium vivax* and *falciparum*, Horn of Africa, 2019

Characteristic	Habitats, no.	Mosquito larvae, no. larvae detected/no. habitats sampled (%)	Mosquito pupae, no. pupae detected/no. habitats sampled (%)
Localities (kebeles) within the town of Awash Sebat Kilo			
Sebat Kilo	60	44/60 (73.3)	19/44 (43.2)
Lemlefan	17	12/17 (70.6)	0/12 (0)
Alalamo	8	8/8 (100.0)	5/8 (62.5)
Artificial containers			
Permanent	48	41/48 (85.4)	17/41 (41.5)
Temporary	37	23/37 (62.2)	7/23 (30.4)
Shade status			
Fully	22	14/22 (63.6)	6/14 (42.9)
Partial	24	22/24 (99.7)	7/22 (31.8)
None	39	28/39 (71.8)	11/28 (39.3)
Use status			
In use	71	54/71 (76.1)	20/54 (37.0)
Not in use	14	10/14 (71.4)	4/10 (40.0)
Container material			
Fiber jar/tire	23	10/23 (43.5)	4/10 (40.0)
Metal/steel tanks/drum/barrel	17	16/17 (94.1)	5/16 (31.3)
Cement/ceramic	45	38/4 (84.4)	15/38 (39.5)
Water turbidity			
Clean	56	45/56 (80.4)	17/45 (37.8)
Turbid	28	19/28 (67.9)	7/19 (36.8)

Table 2. Characteristics of blood meals and mosquito feeding outcomes in study of *Anopheles stephensi* mosquitoes as vectors of *Plasmodium vivax* and f*alciparum*, Horn of Africa, 2019*

Characteristic	*Plasmodium* species		
	P. vivax, n = 36	*P. falciparum*, n = 7	Mixed, n = 4
Parasites/µL, median (IQR)	7,783 (3,603–13,440)	2,431 (867–8,756)	4,516 (1,589–10,563)
Gametocyte positivity, no. positive/no. sampled (%)	25/34 (73.5)	1/7 (14.3)	1/4 (25.0).
Infectious feeds, no. positive/no. sampled (%)	26/36 (72.2)	1/7 (14.3)	2/4 (50.0)
Infected *An. stephensi* mosquitoes, no. positive/no. sampled (%)	446/849 (52.5	2.2	36/104 (34.6)
Infected *An arabiensis* mosquitoes, no. positive/no. sampled (%)	452/1,000 (45.2)	18/200 (9.0)	45/122 (36.9)
Oocysts in infected *An. arabiensis* mosquito midgut, mean (range)	22.8 (1–115)	NA	3.1 (1–22)
Oocysts in infected *An. stephensi* mosquito midgut, mean (range)	24.1 (1–105)	NA	2.8 (1–13)

*Parasite and gametocyte densities were determined by microscopy; IQR, Interquartile range; NA, not available.

Asia (Appendix, https://wwwnc.cdc.gov/EID/article/27/2/20-0019-App1.pdf). The COI tree was more resolutive, suggesting that *An. stephensi* mosquitoes from Ethiopia were most closely related to mosquitoes from Djibouti (64%) and Pakistan (54%).

We conducted 47 paired-membrane feeding experiments by using blood from patients with microscopy-confirmed *P. vivax* or *P. falciparum* infection (Table 2). The proportion of blood-fed mosquitoes was generally higher for *An. arabiensis* (median 80.5%; interquartile range [IQR] 72.5–85.0) than *An. stephensi* mosquitoes (median 53.5%, IQR 44.0–68.0; p<0.001; Figure 1, panel A). The proportions of the 2 mosquito species infected with *P. vivax* were strongly associated (ρ = 0.82, p<0.001; Figure 1, panel B); a significantly higher proportion of *An. stephensi* (median 75.1%, IQR 60.0–85.9) than *An. arabiensis* mosquitoes were infected (median 58.4%, IQR 40.0–85.6; p<0.042). Allowing for the number of dissected mosquitoes for each set of paired feeding experiments, the odds of an individual mosquito becoming infected was higher for *An. stephensi* mosquitoes (odds ratio [OR] 1.99, 95% CI 1.52–2.59; p<0.001) (Figure 1, panel C). The number of oocysts per infected midgut was also higher for *An. stephensi* (median 17, IQR 6–33) than *An. arabiensis* mosquitoes (median 13, IQR 4–30); p<0.001 (Figure 2, panel A). The number of oocysts was positively associated with the proportion of infected mosquitoes for *An. stephensi* (ρ = 0.553, p<0.001) and *An. arabiensis* mosquitoes (ρ = 0.576, p<0.001; Figure 2, panel B). Among paired feedings, sporozoites were detected in 52.2% (47/90) *An. arabiensis* and 75.0% (84/112) *An. stephensi* mosquitoes. A much higher proportion of *An. stephensi* (51.8%, 58/112) than *An. arabiensis*

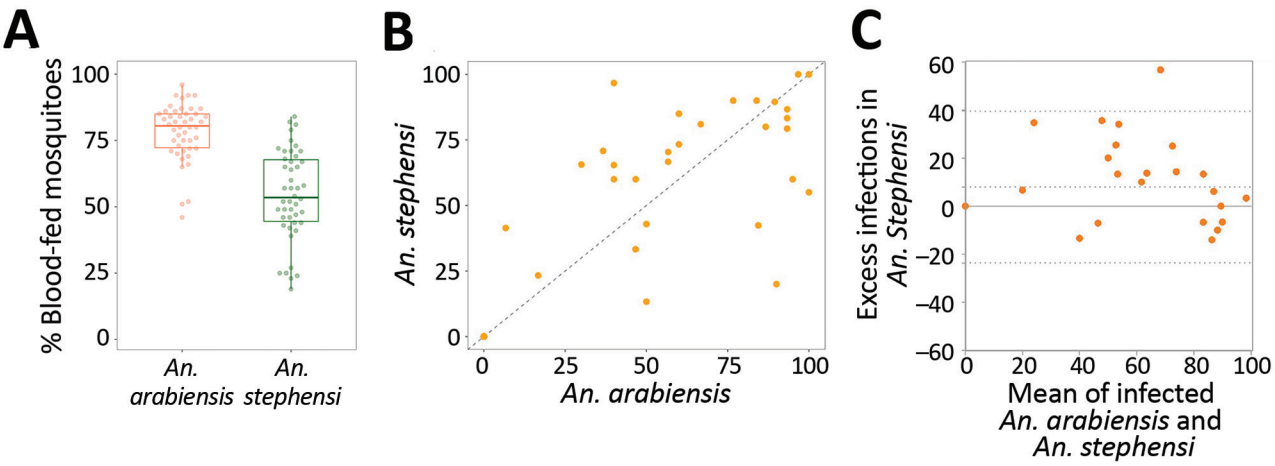

Figure 1. Comparison of feeding efficiency and infection rates for *Anopheles stephensi* and *An. arabiensis* mosquitoes in paired feeding experiments in study of *An. stephensi* mosquitoes as vectors of *Plasmodium vivax* and f*alciparum*, Horn of Africa, 2019. A) Percentage of fully fed *An. arabiensis* mosquitoes (red) and *An. stephensi* mosquitoes (green). Box plots indicate median (midline), 25th (lower line), and 75th (upper line) percentiles of proportion of blood-fed mosquitoes. Whiskers indicate lower and upper 25% scores. Vertical lines indicate minimum and maximum values. B) Percentage of infected mosquitoes. C) Bland-Altman plot (difference plots) for mosquito infection rates in different mosquito species. Symbols indicate differences in infection rates in *An. stephensi* versus *An. arabiensis* (y-axis) mosquitoes in relation to mean infection rates in these 2 species (x-axis). Positive values (57.1%; 16/28) indicate a higher infection rate in *An. stephensi* mosquitoes; dotted lines indicate the 95% limits of agreement. There was no evidence that the correlation coefficient between the paired differences and means differed significantly from 0 (Pitman test of difference in variance, r = 0.026, p = 0.864).

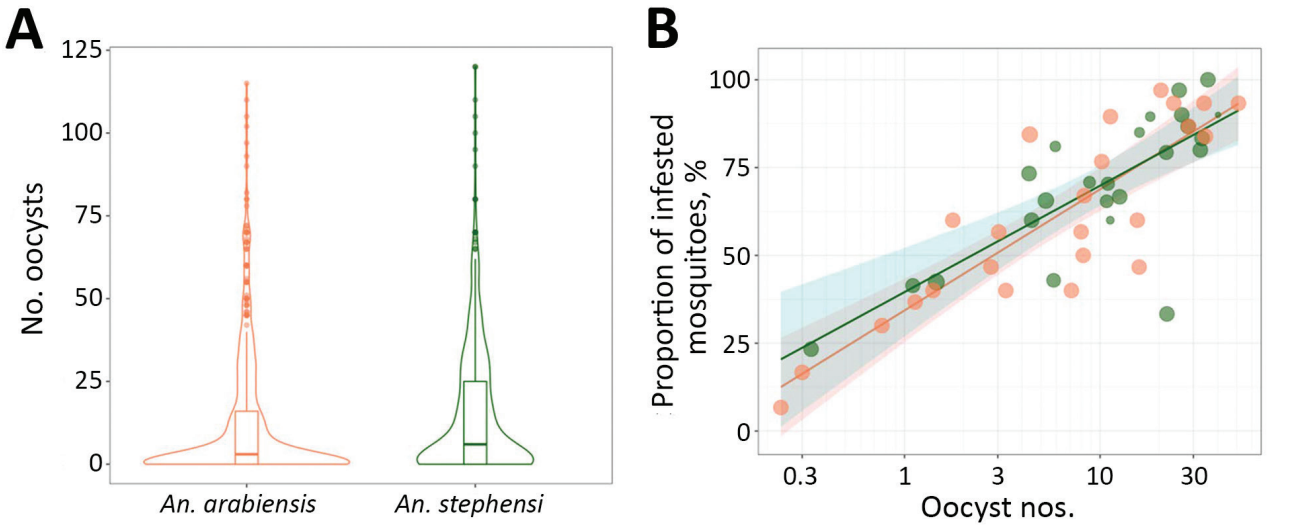

Figure 2. Comparison of relative oocyst numbers and infection rate for *Anopheles stephensi* and *An. arabiensis* mosquitoes in paired feeding experiments in study of *An. stephensi* mosquitoes as vectors of *Plasmodium vivax* and *falciparum*, Horn of Africa, 2019. Number of oocysts per infected midgut for individual mosquitoes of each of the 2 species. A) Violin plot showing estimated kernel density. Horizontal lines indicate median; box indicates interquartile range; and spikes indicate upper and lower adjacent values. The proportion of midguts with detectable oocysts (y-axis) is indicated in association with $\log_{10}$ transformed oocyst numbers (x-axis) for *An. stephensi* (green dots) and *An. arabiensis* (orange dots) mosquitoes. B) Data for 24 feeding experiments in which 723 *An. arabiensis* and 643 *An. stephensi* mosquitoes were dissected. Shaded area indicates 95% CI around estimates for *An. stephensi* (green) and *An. arabiensis* (orange) mosquitoes.

mosquitoes (31.1%, 28/90) had high sporozoite load (+3 and +4); p = 0.011. After accounting for the number of examined salivary glands, the odds of detecting high sporozoite intensity were substantially higher for *An. stephensi* than *An. arabiensis* mosquitoes (OR 4.6, 95% CI 2.2–9.9; p<0.001).

Conclusions

An. stephensi mosquitoes have spread from Asia throughout the Horn of Africa, detected in Djibouti in 2012 (*11*), Ethiopia in 2016 (*12*), and Sudan in 2019 (*3*). The widescale presence of *An. stephensi* mosquitoes in developmental stages in artificial water bodies demonstrates that these mosquitoes are firmly established in an urban setting in Ethiopia, located on the main transportation corridor from Djibouti to Addis Ababa. Detection of 4 haplotypes suggests independent arrival of different populations or heterogeneity arising after importation of the mosquito species. Our mosquito feeding experiments predominantly included highly infective patients with clinical *P. vivax* infection (*10*,*13*). Although feeding rates for the membrane-adapted colony of *An. arabiensis* mosquitoes were high, mosquito infection rates were significantly higher for *An. stephensi* mosquitoes. Our detection of salivary gland sporozoites establishes that sporogonic development of local *P. vivax* can be completed by *An. stephensi* mosquitoes. We recruited fewer patients with clinical *P. falciparum* infection, who were less likely than *P. vivax* patients to infect mosquitoes (*10*). Despite a modest number of observations, our findings demonstrate that local *P. falciparum* isolates are also capable of infecting *An. stephensi* mosquitoes and are further supported by detection of *P. falciparum*– and *P. vivax*–infected wild-caught adult mosquitoes.

Spread of *An. stephensi* mosquitoes poses risk for increased *P. falciparum* and *P. vivax* receptivity and local transmission in urban Africa. Given mosquito preference for human-made containers (*14*), our findings support integrated vector management recommended by the World Health Organization under the Global Vector Control Response (*15*). Management may include integrated surveillance and control of other vectors such as *Aedes aegypti* mosquitoes for larval source management.

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Appendix

Aquatic habitat characterization

A total of (n = 85) potential larval habitats were surveyed in 5 days (May 2019) in Awash Sebat Kilo town (Appendix Figure 1) using a standardized check list/questionnaire (22 question sets) to capture important characteristics. Immatures were collected throughout the day. Altitude, latitude, longitude, sun light exposure, water turbidity, substrate type, presence of vegetation, predators, and competitors were recorded for each site. The *Anopheles* larvae were separated from culicine larvae and classified as early- (1st, 2nd) or late-instars (3rd and 4th) stage and larval density was recorded by instars. The *Anopheles* larvae/pupae were transported to Adama malaria center with jars and transferred to larval tray for rearing to adult using the same filtered water from the breeding site. Detailed outcome of the aquatic habitat survey can be obtained using this link (https://datadryad.org/stash/dataset/doi:10.5061/dryad.gf1vhhmnt; Table 1). Aquatic habitat characterization) with number of larvae/pupae collected in each of the 10 dips (https://doi.org/10.5061/dryad.gxd2547hr; Table 2_Larvae pupae density).

Adult mosquitoes resting, biting and host preference behavior

Resting, feeding and host preference behavior of *An. stephensi* was assessed using 5 entomological sampling techniques: i) Centers for Disease Control (CDC) light trap, ii) human landing catches (HLC), iii) pyrethrum spray sheet collection (PSC), iv) aspiration from animal shelters, and v) cattle baited traps. The CDC light traps (Model 512; John W. Hock Company, Gainesville, FL, USA) were set 1 m above the ground on a wall or roof, both indoors and

outdoors on 15 randomly selected households for two nights that makes a total of 60 traps in 30 nights. Indoor traps were hung at the foot edge of the person who slept under an untreated bed net (*1*). Other occupants in the houses were left to use LLINs provided by the control program as part of the routine malaria control. The traps were switched on at 6:00PM and off at 6:00AM the next morning in each sampling night. PSC were conducted from 6:00 AM to 2:00 AM on five randomly selected households' per-day and 20 households were included in each round of sampling, thus a total of 60 households were sampled in three rounds. The HLC were conducted in nine selected households both in and outdoors that was repeated the next day. Locally trained entomology technicians were employed to collect female *Anopheles* by standard mouth aspirator from 6:00 PM to 6:00 AM from both indoors and outdoors. Two collectors were assigned at a time for each house (one outdoors and one indoors) in shifts of 6 hours (the first shift being 6:00PM – 12:00PM and the second from 12:00 PM to 6:00 AM). Collectors in the same shift changed with each other between outdoors and indoors every hour after recording their findings on the checklist to avoid bias due to individual variation in attraction and competence. In addition to this, animal sheds were inspected using HC and cattle bait trap (*2*) were conducted for collecting mosquitos biting and resting in animal shelters. Mosquitoes resting in animal shelters and cattle bait traps were collected using standard mouth aspirator for 30 minutes in each, from 5:30 AM to 6:00 AM. All collected *Anopheles* mosquitoes were counted and sorted out morphologically to species level (*3*,*4*) and by their abdominal stage into unfed, freshly fed, half-gravid or gravid (*5*), except those collected by HLC. In animal shelter with high number of mosquito collection was repeated the next morning.

Of the five methods used for mosquito collection in the 2 monthly studies of 6 days each and therefore 12 days in total, *Anopheles* were caught only by the three methods: CDC light traps, aspiration from animal shelter (hand collection), and human landing catches. This makes it an average of 2.5 female *Anopheles* caught per trap per night. Detailed survey results for different adult catch methods is provided using this link (https://datadryad.org/stash/dataset/doi:10.5061/dryad.gf1vhhmnt; Table 3_Adult mosquitoes surveillance).

Optimization of feeding efficiency of adult *An. stephensi* raised from wild collected larvae/pupae

Optimum starvation time for the *An. stephensi* raised from wild collected larvae and pupae was assessed in three different experiments with 2, 3, 4 or 5 hours before feeding evaluated in each experiment (Appendix Table 1).

Appendix Table 1. Feeding optimization for *Anopheles stephensi* raised from wild collected larvae/pupae

Code of Experiment	Starvation hour prior feeding	Number of fed mosquitoes	Feeding efficiency, % of blood fed
E01–01	5	58	39.2
E01–02	4	68	45.9
E01–03	3	85	53.8
E01–04	2	86	50.3
E02–01	5	24	36.4
E02–02	4	31	42.5
E02–03	3	29	44.6
E02–04	2	17	28.3
E03–01	5	33	36.2
E03–02	4	46	48.9
E03–03	3	44	51.6
E03–04	2	35	35.0

Molecular detection of parasites and blood meal sources and targeted sequencing of morphologically identified *An. stephensi* mosquitoes

Plasmodium infection status of individual wild-caught morphologically-confirmed adult *An. stephensi* mosquitoes was assessed using nested polymerase chain reaction (nPCR) targeting the small 18S subunit (*6*) using genomic DNA extracted from homogenate of mosquito's head-thorax and abdomen separately (*7*), indicating sporozoite and oocyst-stage infections, respectively. Multiplex PCR that targets the mitochondrial cytochrome b gene and produces species-specific fragments of varying sizes was used to assess blood meal sources of individual mosquitoes (*8*). For confirmation of morphologically identified *An. stephensi*, DNA was extracted from whole mosquito bodies using the DNeasy Blood and Tissue kit (Qiagen, UK). PCR was performed for each individual mosquito, targeting the nuclear internal transcribed spacer 2 region (ITS2) and the mitochondrial cytochrome oxidase subunit 1 gene (COI) (*9*). Following PCR clean-up (Source BioScience Plc, Nottingham, UK), chain termination sequencing was performed to generate unambiguous consensus sequences for each sample (Supplemental notes). Sequences were assembled manually in BioEdit v7.2.5 (*10*) to create unambiguous consensus sequences for each sample. Consensus sequence alignments per gene were generated in ClustalW and used to perform nucleotide BLAST (NCBI) database queries (*11*). *An. stephensi* ITS2 and COI sequences, from across the vector's geographic range, were

downloaded from GenBank for phylogenetic analysis in MEGA X (*12*). Additional outgroup ITS2 sequences were retrieved for *An. maculatus*, *An. maculipalpis*, *An. sawadwongporni* and *An. willmori.* Alternate maximum-likelihood (ML) phylogenies were constructed using the Jukes-Cantor (ITS2; final tree *ln*L = −916.913) or Tamura-Nei (COI; final tree *ln*L = −732.248) models, following appropriate nucleotide substitution model selection in MEGA X. Bootstrap support for clade topologies was estimated following the generation of 1,000 pseudoreplicate datasets. As indicated in Appendix Figure 2. *An. stephensi* from Ethiopia are more related with those from Pakistan and Djibouti.

Sporozoite quantification

Sporozoites were quantified on day 12 post feeding in salivary glands of mosquitoes that remained from the batch where high oocysts were detected during midgut dissection on day 7 post feeding and categorized into four (with a grade from 1–4) following protocol reported before (*14*). Table 4_Sporozoite quantification, following this link (https://datadryad.org/stash/dataset/doi:10.5061/dryad.gf1vhhmnt) depicts the detail results for each *An. arabiensis* and *An. stephensi* dissected (rows) with representative pictures in Appendix Figure 3, panels A, B.

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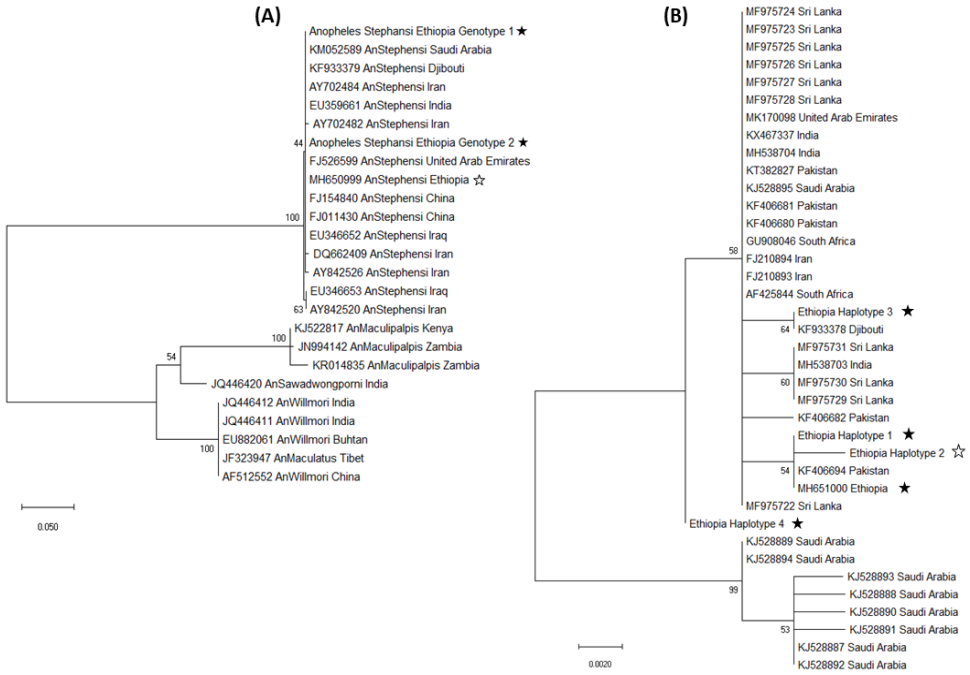
Appendix Figure 1. *Anopheles. stephensi* larval habitats. Images are of waterbodies that were infested with developmental stages of *An. stephensi,* namely water reservoirs made of bricks or cemented tanks (A – B), metal (C and D), barrels (E – F), plastic (G), or fiber (H). The median volume of the aquatic containers was 4m^3 (interquartile range, 1.0–15.6) and ranged from 0.06 m^3 to 360 m^3. The majority of the containers were uncovered and were in use for household (32) and construction purposes (34). The material from which the different types of reservoirs were made of included cement (n = 45), plastic (n = 9), fiber (n = 14) and steel (n = 17).



Appendix Figure 2. Maximum-likelihood phylogenies of ITS2 (left) and COI (right). Maximum-likelihood topologies were constructed using representative reference sequences with published geographic data downloaded from GenBank. Within the Ethiopian population, due to the presence of a hyper-variable

microsatellite region, ITS2 sequences (A) were trimmed to create a consensus alignment of 289 bp; one polymorphic site separated samples into two genotypes (indicated with filled asterisk together with the previously reported genotype, MH650999, Carter, et al. (*13*) in unfilled asterisk). COI sequences (B) were assembled into a consensus alignment of 687 bp; a total of four variable sites were identified, corresponding to four haplotypes (indicated with filled asterisk together with the previously reported genotype, MH651000, Carter, et al. (*13*), unfilled asterisk). Nucleotide sequences for ITS2 and COI were deposited in GenBank under the following accession numbers: Ethiopia Genotype1, MN826065; Ethiopia Genotype2, MN826066; Ethiopia Haplotype1, MN826067; Ethiopia Haplotype2, MN826068; Ethiopia Haplotype3, MN826069; and Ethiopia Haplotype4, MN826070. Scale bars indicate nucleotide substitutions per site.