An Update on the Distribution, Bionomics, and Insecticide Susceptibility of Anopheles Stephensi in Ethiopia, 2018-2020

Meshesha Balkew
ABT Associates Inc
Peter Mumba
USAID
Gedeon Yohannes
ABT Associates Inc
Ephrem Abiy
ABT Associates Inc
Dejene Getachew
Dire Dawa University
Solomon Yared
Jijiga University: Jigjiga University
Amha Worku
Jijiga University: Jigjiga University
Araya Gebresilassie
Addis Ababa University
Fitsum G Tadesse
Armauer Hansen Research Institute
Endalamaw Gadisa
Armauer Hansen Research Institute
Endashaw Esayas
Armauer Hansen Research Institute
Temesgen Ashine
Armauer Hansen Research Institute
Delenasaw Yewhalaw
Jimma University
Sheleme Chibsa
USAID
Hiwot Teka
USAID
Matt Murphy
Centers for Disease Control and Prevention
Melissa Yoshimizu
USAID
Dereje Dengela
ABT Associates Inc
Sarah Zohdy
Centers for Disease Control and Prevention
Seth R. Irish (xjs7@cdc.gov)
Centers for Disease Control and Prevention

Research

Keywords: bionomics, Anopheles stephensi , malaria vector, enzyme linked immunosorbent assays (ELISA)

Posted Date: May 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-499226/v1

Abstract

Background

Anopheles stephensi, an invasive malaria vector, was first detected in Africa nearly 10 years ago. After the initial finding in Djibouti, it has subsequently been found in Ethiopia, Sudan, and Somalia. To better inform policies and vector control decisions, it is important to understand the distribution, bionomics, insecticide susceptibility, and transmission potential of *An. stephensi*. These aspects were studied as part of routine entomological monitoring in Ethiopia between 2018 and 2020.

Methods

Adult mosquitoes were collected using human landing collections, pyrethrum spray catches, CDC light traps, animal-baited tent traps, resting boxes, and manual aspiration from animal shelters. Larvae were collected using handheld dippers. The source of blood in bloodfed mosquitoes and the presence of sporozoites was assessed through enzyme linked immunosorbent assays (ELISA). Insecticide susceptibility was assessed for pyrethroids, organophosphates, and carbamates.

Results

Adult *An. stephensi* were collected with aspiration, black resting boxes, and animal-baited traps collecting the highest numbers of mosquitoes. Although sampling efforts were geographically widespread, *An. stephensi* larvae were collected in urban and rural sites in eastern Ethiopia, but *An. stephensi* larvae were not found in western Ethiopian sites. Blood meal analysis revealed a high proportion of blood meals that were taken from goats, and only a small proportion from humans. *Plasmodium vivax* was detected in wild collected *An. stephensi*. High levels of insecticide resistance were detected to pyrethroids, carbamates, and organophosphates. Pre-exposure to piperonyl butoxide increased susceptibility to pyrethroids. Larvae were found to be susceptible to temephos.

Conclusions

Understanding the bionomics, insecticide susceptibility, and distribution of *An. stephensi* will improve the quality of a national response in Ethiopia and provide additional information on populations of this invasive species in Africa. Further work is needed to understand the role that *An. stephensi* will have in *Plasmodium* transmission and malaria case incidence. While additional data are being collected, national programs can use the available data to formulate and operationalize national strategies against the threat of *An. stephensi*.

Introduction

Anopheles stephensi is one of the primary vectors of malaria in Asia (Sinka et al. 2011). In 2012, *An. stephensi* was found in Djibouti, marking the first confirmed report of this malaria vector from the African continent (earlier reports of *An. stephensi* in Egypt were later determined to be *Anopheles ainshamsi*) (Faulde et al. 2014; Gad et al. 2006). In 2016, *An. stephensi* was found in the Somali Region in eastern Ethiopia (Carter et al. 2018). Since then, *An. stephensi* has been found in an increasing number of sites in eastern Ethiopia (Balkew et al. 2020), Sudan (WHO 2020), and Somalia (WHO 2020).

The spread of this vector is a grave concern for malaria control and elimination in the Horn of Africa, as data from Djibouti indicate the presence of *An. stephensi* has been associated with dramatic increases in malaria cases (Seyfarth et al. 2019). Suspected and confirmed malaria cases in Djibouti have increased nearly 30-fold—from 1,684 in 2012 to 49,402 in 2019 (WHO 2020). While similar increases have not been yet reported in Ethiopia, recent work has shown that *An. stephensi* is a competent vector of *Plasmodium vivax* there (Tadesse et al. 2021). While *Anopheles arabiensis* remains the primary vector of malaria in Ethiopia, the threat of the spread of this vector species, occupying a different ecological niche, is a major concern.

To improve understanding of the spread of *An. stephensi* in Ethiopia, regular sampling was conducted from 2018 to 2020. In addition, the bionomics and insecticide resistance status of *An. stephensi* was studied through routine surveillance and insecticide resistance monitoring activities. While some collection data from 2018 has been presented elsewhere (Balkew et al. 2020), subsequent results are presented here, with the primary aim to guide the Ethiopian National Malaria Elimination Program (NMEP) in implementing effective vector surveillance and control measures against this invasive mosquito species.

Materials And Methods

Study sites

In order to determine the distribution of *An. stephensi*, field surveys using one-time larval collections and identification of adults from reared larvae were conducted in 21 urban sites in Ethiopia in 2018 and 2019. In 2020, field surveys were expanded into peri-urban and rural sites within 20 km radius of 11 urban areas where *An. stephensi* had been previously collected. Adult mosquito collections were made in 10 sites in 2018, and 4 sites in 2019 and 2020. All sites where *An. stephensi* surveys were conducted are shown in Figure 1 and described in Table 1.

Mosquito collection

Collection of larvae and pupae

Larvae and pupae were sampled in each site through a survey conducted by a team of three collectors who inspected the urban areas on foot, sampling all visible bodies of standing water and water-holding containers. Surveys generally lasted 6-7 days per site; all mosquito larvae collected were reared to adults for identification using a morphological key (Coetzee 2020). The survey teams were guided by staff with local knowledge of the area; surveys were not systematically conducted. GPS points of survey sites were not recorded until 2020. In 2020, to investigate whether *An. stephensi* had spread outside of urban areas into peri-urban and rural areas, larval collections were made in peri-urban areas or villages within 20 km of urban areas where *An. stephensi* had been found. Additionally, in 2020, the presence of *Aedes* larvae (generally *Aedes aegypti*) was also recorded in the surveyed sites. In addition to larvae collected to determine the distribution of *An. stephensi*, larvae were also collected and reared to adults for insecticide susceptibility tests in 2018 (two sites), 2019 (five sites), and 2020 (four sites) (see below).

Collection of adult mosquitoes

Longitudinal surveillance of adult *An. stephensi* took place in Dire Dawa and Kebridehar from June to December 2019 and in Awash Sebat Kilo and Metehara towns from August to December 2019 (rainy season). The following methods were used in each site each month: human landing collections (HLC) (6 indoor and 6 outdoor collection nights), pyrethrum spray catches (PSC) (20 houses), CDC light traps (12 indoors and 12 outdoors), animal-baited tent traps (3 nights), and manual aspiration from animal shelters (2-20 collections per site). Additionally, black resting boxes (6 nights) were placed outdoors in the same compounds of HLC houses in Dire Dawa and Kebridehar, and near a horse stable in Dire Dawa.

Human landing collections were conducted indoors and outdoors at the same houses each month between 1800h and 600h. Mosquito collectors caught mosquitoes using mouth aspirators and placed them in labelled paper cups covered with mosquito netting. All mosquitoes collected each hour were aspirated into the same paper cup. Each hour, the collectors swapped positions between indoor and outdoor. If collectors showed symptoms of malaria, they were referred to health centers for free consultation and treatment.

Pyrethrum spray collections were conducted between 600h and 900h. Any structural gaps in the house were blocked and any food or cooking utensils and domestic animals were removed from the house. White sheets were spread on the floor of each room inside the house and a commercially available pyrethroid aerosol spray was sprayed inside the house. The house was closed for 10 minutes and then the sheets were individually carried outside and inspected for knocked-down mosquitoes.

CDC light traps (Bioquip, Rancho Dominguez, CA, USA) were set each day between 1600h and 1900h. The indoor traps were suspended at 1.5m at the foot of a bed, with residents of the household sleeping under their own insecticide-treated nets (if nets were not available, they were provided). Mosquitoes were retrieved from each trap the following morning between 600h and 900h. Outdoors, a temporary shelter at a distance of 10 meters from the same house was constructed and a volunteer slept on a camp bed protected by a treated net. A trap was hung on a pole 1.5m above the ground by the feet of the volunteer.

Animal-baited tent traps were composed of a tethered ox, cow, or goat under an untreated tent raised off the ground by 5cm to allow mosquitoes to enter. The animal was kept inside the tent from 1800h and resting mosquitoes on the wall of the tent were collected the following morning between 600h and 700h. Any mosquitoes present in the tent were collected with a mouth aspirator and put into a paper cup, covered with mosquito netting, until no more mosquitoes were found.

In 2019, manual aspiration was conducted using a mouth aspirator to collect mosquitoes resting in animal shelters but in 2020 this activity was replaced by Prokopack aspirators following the President's Malaria Initiative (PMI) COVID-19 mitigation measures. Generally, horse stables were composed of walls on two sides and fences on two sides, with a corrugated tin roof. Goat and cattle shelters were made of brick walls on all sides with either a corrugated tin or thatched roof. Aspiration was conducted in the shelters between 600h and 900h and each shelter was inspected for 10-15 minutes. Any mosquitoes present were aspirated with a mouth aspirator into a paper cup, covered with mosquito netting while the Prokopack collections were kept in collection cups.

Black resting boxes were constructed of cardboard boxes in which the interior was lined with black nylon cloth. The boxes were placed in the compound of houses assigned for HLCs and horse stables before 1800h and were inspected for the presence of mosquitoes the following morning between 600h and 700h. Any mosquitoes present in the boxes were collected with a mouth aspirator into a paper cup covered with mosquito netting, until no more mosquitoes were found.

Anopheles mosquitoes were identified morphologically to species using a key by Coetzee (2020) and stored individually in Eppendorf tubes with silica gel for laboratory processing.

Blood meal analysis

The abdomens of blood-fed mosquitoes collected in 2019 from Dire Dawa and Kebridehar were subjected to a direct Enzyme Linked Immunosorbent Assay (ELISA) following the method described in Beier *et al.* (1988). Briefly, a homogenate of each specimen was prepared in 50µL of phosphate buffer saline (PBS) and transferred into an individual well of a 96-well assay plate and incubated for three hours. For each wash step, 200µL of PBS-Tween (0.5% Tween 20 in PBS) was used. The wells were washed twice and then incubated with 50µl of conjugate per well for one hour. The conjugate was incubated for three hours at 4°C before use and consisted of host-specific peroxidase-labeled monoclonal antibody of human, bovine, goat or dog. Positive and negative controls included whole blood samples collected from each host and non-blood-fed insectary-reared *An. arabiensis*, respectively. The total volume in the wells was removed by aspiration, and the plate was washed three times and incubated with 100µl ABTS for 30 minutes. Following incubation, absorbance was immediately measured using a spectrophotometer at 405 nm (ELX800, BioTek, Winooski, VT, USA).

Detection of Plasmodium sporozoites

All adult mosquitoes collected during longitudinal monitoring in Dire Dawa and Kebridehar in 2019 were tested for presence of sporozoites, using circumsporozoite (CS) ELISA. The heads and thoraces from all morphologically identified *An. stephensi* were assayed to detect antibodies against the circumsporozoite proteins of *Plasmodium falciparum* (Pf), *P. vivax*-210 (Pv-210), and *P. vivax*-247 (Pv-247), using the sandwich CS-ELISA according to the protocol established by Wirtz *et al.* (1992). At least four negative controls and four positive controls were used for each ELISA plate. The cutoff value for the CS-ELISA was determined as two times the mean absorbance value of negative samples. Positive samples were not boiled and retested.

Insecticide susceptibility tests

Susceptibility

Insecticide susceptibility tests were conducted on adult *An. stephensi* reared from wild larvae from two sites in 2018 (Dire Dawa, and Kebridehar), five sites in 2019 (Awash Sebat Kilo, Dire Dawa, Gewane, Kebridehar, and Semera), and four sites in 2020 (Awash Sebat Kilo, Godey, Meki, and Metehara) following standard procedures (WHO, 2016). Seventy-five to 100 mosquitoes from each population were tested for each insecticide and 50 were used for controls. The insecticides used were 0.1% bendiocarb, 0.1% propoxur, 0.25% pirimiphos-methyl, 0.05% alpha-cypermethrin, 0.05% deltamethrin, and 0.75% permethrin.

Larval susceptibility assays were conducted in November 2020 to determine the susceptibility of *An. stephensi* larvae to temephos, an organophosphate larvicide. Larvae from five sites (Awash Sebat Kilo, Dire Dawa, Kebridehar, Meki, and Semera) were tested. Assays were conducted according to an established protocol (WHO, 2005). Briefly, temephos was added to cups of tap water to produce 250ml volumes of concentrations ranging from 0.125 to 0.00375mg/L, to calculate the concentration killing 50% and 95% of larvae. The estimated diagnostic dose of 0.25mg/L was used to indicate resistance (WHO, 1981). Approximately 25 third-instar larvae of *An. stephensi* were added to cups and mortality was recorded 24 hours later. Four cups were used per dose to achieve 100 larvae tested per dose. Larvae and pupae collected from the same habitat were raised to adults for species identification to confirm *An. stephensi*.

PBO synergist assays

In 2018, piperonyl butoxide (PBO) synergist assays were conducted on *An. stephensi* from Dire Dawa and Kebridehar against two pyrethroids (deltamethrin and permethrin). In 2019, PBO synergist assays were conducted against three pyrethroids (alpha-cypermethrin, deltamethrin, permethrin) in Dire Dawa and against deltamethrin in Awash Sebat Kilo. In 2020, synergist assays were conducted against the same three pyrethroid insecticides in Awash Sebat Kilo, Godey, Meki, and Metehara. The synergist assays were conducted by pre-exposing mosquitoes to a 4% PBO paper for 60 minutes. Mosquitoes were then transferred to tubes with the pyrethroid of interest for 60 minutes and the susceptibility was determined as described for adult susceptibility tests described above.

Resistance intensity

In Awash Sebat Kilo (2019, 2020), Meki (2020), and Metehara (2020), the resistance intensity of *An. stephensi* to alpha-cypermethrin, deltamethrin, and permethrin was assessed through exposure to 1x, 5x, and 10x the diagnostic dose. Mosquitoes were exposed to the insecticides for 60 minutes, and susceptibility assessed according to procedures described above.

Results

Distribution of Anopheles stephensi

In 2019, *Anopheles stephensi* were found in three of the 11 urban sites where larval surveillance was conducted (Figure 1). In Meki, larvae were found in tires, concrete water containers, water tanks, and discarded buckets. In Metehara, larvae were collected from water tanks. In Zeway, larvae were found in tires, water drums, and concrete water containers. Other *Anopheles* larvae collected included *An. gambiae* s.l., *An. rhodesiensis*, and *An. cinereus* (Table 2). *Anopheles stephensi* was not detected in the towns of Assosa, Bahirdar, Gambela, Hawassa, Jimma, Negelle-Borena, Shire, and Yabello.

In 2020, to determine whether *An. stephensi* was present in rural areas, kebeles (rural and peri-urban villages) within 20km of an urban site were searched for larvae. *Anopheles stephensi* was found in 21 of the 55 kebeles investigated. The results of these searches are shown in Table 3. Larval sites in which *An. stephensi* were found included: water drums, plastic water tanks, puddles, concrete wells, plastic sheets, discarded tires, flooded cement floors of a house under construction, and metal water tanks (Supplementary Table 1). In 40% of the sites where *An. stephensi* were found, *Aedes* larvae were also collected (Table 3).

Anopheles stephensi in longitudinal surveillance sites

A total of 1,040 adult *An. stephensi* were collected from Dire Dawa (n=412), Kebridehar (n=368), Awash Sebat Kilo (n=154), and Metehara (n=106) in 2019 (Table 4). The majority (n=585, 56.3%) were collected in animal shelters (cattle, goats, sheep, and horses) using manual aspiration. In the peri-urban areas of Dire Dawa, nearly 39% (n=159) of *An. stephensi* collected were found resting in black boxes placed in the compounds of houses with horse stables. Black resting boxes were not effective in compounds without horse stables. Cattle-baited traps caught 19.0% (n=198) of all *An. stephensi* collected. The most

common mosquito sampling methods, PSC, HLC, and CDC light trap, were less effective than aspiration, black boxes, and animal-baited traps in collection of adult *An. stephensi*. The greatest numbers of *An. stephensi* were collected in August, September, and October.

Blood meal identification

A total of 631 visibly-bloodfed *An. stephens*i from Dire Dawa and Kebridehar sites, collected in 2019, were tested by ELISA for blood meal sources. One (0.25%) of the 394 *An. stephensi* from Dire Dawa and 0/237 from Kebridehar were found with human blood only. In contrast, 29.7% and 53.2% were found to have fed on goats alone, and 1.02% and 0.4% on cows alone, in the respective sites. Dog blood alone was the source of 2.03% of bloodmeals of *An. stephensi* from Dire Dawa and 1.3% from Kebridehar. Mixed blood was found in 20.9% of *An. stephensi* tested. The remaining 38.4% of blood meals were not identified. The frequency of bloodmeals from each source is provided in Figure 2.

Plasmodium falciparum and P. vivax infection with sporozoites

All of the 780 adult *An. stephensi* specimens (412 from Dire Dawa and 368 from Kebridehar) were tested for *P. falciparum* and *P. vivax* circumsporozoite proteins. Of these, three were positive for *P. vivax*, with infection rates of 0.5% and 0.3% from Dire Dawa and Kebridehar, respectively. The two positive samples from Dire Dawa were of the Pv-210 variant and the single positive sample from Kebridehar was of the Pv-247 variant. None of the tested *An. stephensi* were positive for *P. falciparum*.

Insecticide susceptibility

In 2018, in both Dire Dawa and Kebridehar, *An. stephensi* was found to be resistant to all pyrethroids and carbamates tested and was only susceptible to pirimiphos-methyl (Table 5). Pre-exposure of mosquitoes to PBO increased susceptibility of *An. stephensi* to deltamethrin to 96% in Dire Dawa. PBO pre-exposure fully restored susceptibility (100% mortality) to both deltamethrin and permethrin in Kebridehar.

In 2019, *An. stephensi* from all five sites were highly resistant to bendiocarb, alpha-cypermethrin, deltamethrin, and permethrin (Table 5). *An. stephensi* were susceptible to propoxur and pirimiphos-methyl in only one site, Semera (99% mortality for both insecticides), and resistant to pirimiphos-methyl in Dire Dawa and Kebridehar. Possible resistance to pirimiphos-methyl was recorded in Awash Sebat Kilo and Gewane. In the synergist assays, pre-exposure to PBO restored full susceptibility to alpha-cypermethrin and permethrin in Dire Dawa and to deltamethrin in Awash Sebat Kilo, and substantially increased susceptibility to deltamethrin (up to 97% mortality) in Dire Dawa.

In 2020, *An. stephensi* resistance to bendiocarb, propoxur, pirimiphos-methyl, and the three pyrethroids (deltamethrin, permethrin, and alpha-cypermethrin) was observed in the four sites tested (Table 5). When *An. stephensi* were pre-exposed to PBO before exposure to pyrethroids, susceptibility was fully restored to permethrin in all four sites, to deltamethrin in 3/4 sites, and to alpha-cypermethrin in 2/4 sites (Table 5).

Resistance intensity in Awash Sebat Kilo in 2019 and in Awash Sebat Kilo, Meki, Metehara, and Godey in 2020 is shown in Figure 3. At the diagnostic dose, resistance was found to all three pyrethroids in all locations and years, with the exception of Metehara in 2020, where possible resistance (93% mortality) was found to permethrin. Resistance to alpha-cypermethrin, even at 10x the diagnostic dose, was found in all sites and years. For deltamethrin, resistance or possible resistance was found at either the 5x level (Awash Sebat Kilo 2019 and Meki 2020) or 10x level (Awash Sebat Kilo 2020), however, even at 10x the diagnostic dose, *An. stephensi* in Metehara 2020 remained resistant to deltamethrin. Susceptibility to permethrin was found at 5x (Awash Sebat Kilo 2020 and Meki 2020) or 10x (Awash Sebat Kilo 2019 and Metehara 2020).

Temephos susceptibility test results

All the *An. stephensi* populations tested were found to have 100% mortality at less than the threshold for susceptibility (0.25mg/L). In Awash Sebat Kilo, Dire Dawa, and Kebridehar, 100% mortality was observed at 0.125mg/L. In Meki mortality was 100% at 0.03125mg/L, and in Semera mortality was 100% at 0.0625mg/L. The LC_{50} and LC_{95} values were calculated for three of the sites (Table 6).

Discussion

The larval sites where *An. stephensi* were found in 2019 and 2020 resemble those previously reported (Balkew et al. 2020), such as water storage containers, barrels, and wells. In addition, *An. stephensi* were found in puddles, wells, and the flooded cement floor in a house under construction. In general, the percentage of inspected sites that were positive for *An. stephensi* was low (≤33%). The sites where *An. stephensi* were present often contained *Aedes* larvae, indicating that larval control of these sites might have benefits for prevention of both malaria and *Aedes*-borne diseases.

The highest numbers of adult *An. stephensi* were collected in the four longitudinal monitoring sites with manual aspiration of mosquitoes from animal shelters. Determining the most efficient collection method could not be done, unfortunately, as the number of collections made was not recorded. While the largest numbers of adult *An. stephensi* were collected in August, September, and October, a more rigorous and standardized collection protocol is needed to determine patterns of seasonality. Furthermore, anecdotal reports indicate that *An. stephensi* may be present during the dry season. Determining the most effective collection method and the seasonality of *An. stephensi* remains a priority.

Blood meal analysis revealed high levels of zoophagy particularly on goats; however, host blood meal from a large proportion of samples could not be identified through the ELISA method. This could be due to the blood meal host sources not being represented amongst the reagents used. Additionally, blood meal analysis using PCR could be used to identify species-specific blood meals (Kent & Norris 2005). Since many of the mosquitoes analyzed in this study were collected from horse shelters, adding this host to the blood meal analysis activity is a priority. Nonetheless, the results from this work are in line with blood feeding indices noted in India, where high levels of zoophagy were observed, even in urban settings (Thomas et al. 2017).

In a previous study, *An. stephensi* collected in 2020 in Ethiopia were reared in the lab to determine vectorial capacity (Tadesse et al. 2021). The findings suggested that Ethiopian *An. stephensi* are more competent vectors of *P. vivax* than *An. arabiensis*; however, little is known about sporozoite rates of wild *An. stephensi* in Ethiopia. In this study, *P. vivax* sporozoite rates of 0.5% and 0.3% were found in Dire Dawa and Kebridehar, respectively, though the percentage of human blood meals from the two locations were 0.25% and 0%, respectively. More work is needed to see whether collection bias may have resulted in underestimates of human feeding, or if the vector capacity of *An. stephensi* is efficient enough that even low levels of human feeding result in sporozoite rates similar to wild caught *An. arabiensis* (Tadesse et al. 2021; VectorLink 2019).

Widespread pyrethroid resistance in *An. stephensi* has been reported from Asia (Enyati et al. 2020) and resistance has also been reported in Ethiopia (Yared et al. 2020). However, to combat and control this invasive vector, a fuller understanding of insecticide resistance profiles is necessary. While considerable variation was noted between years in the phenotypic susceptibility assay results, a general pattern of high levels of pyrethroid resistance was evident. Similarly, resistance to the carbamates propoxur and bendiocarb was noted. Resistance to pirimiphos-methyl was more variable, with susceptibility noted in some settings and high levels of resistance detected in others. Resistance to pyrethroids was intense for alpha-cypermethrin and deltamethrin, but less so for permethrin. This resistance appeared to be likely mediated in large part by oxidases, as pre-exposure of mosquitoes to PBO resulted in large increases of mortality.

The implications of resistance patterns are important for vector control decision making. Currently, insecticide treated nets (ITNs) are not distributed and indoor residual spraying (IRS) is not conducted by the NMEP in urban settings due to the documented low risk of malaria (EPHI 2016), resource limitations, and low community acceptance of IRS. New types of nets, such as PBO nets, may be useful vector control tools for use against *An. stephensi*. Further work is needed to understand *An. stephensi* susceptibility to chlorfenapyr and pyriproxyfen, additional insecticides used in bi-treated nets. IRS is largely conducted in rural areas using products containing pirimiphos-methyl and clothianidin, so further work is needed to clarify the susceptibility of *An. stephensi* to these insecticides as well.

Temephos has been used as a larvicide in Ethiopia to control both *An. arabiensis*. All five sites where *An. stephensi* was tested for temephos susceptibility showed complete susceptibility. Further work is needed to determine the susceptibility to other larvicides that might be used for control of *An. stephensi* (e.g. *Bacillus thuringiensis* var. *israelensis*, pyriproxyfen).

More data are needed to determine the distribution, role as a vector of malaria parasites, and interventions that can effectively control *An. stephensi* in Ethiopia. This must be a priority not only for the NMEP in Ethiopia, but for the entire malaria control community. Sinka *et al.* (2020) predicted that an additional 126 million people in Africa might be at risk of contracting malaria if *An. stephensi* spreads throughout Africa. While *An. stephensi* appears to be capable of colonizing urban, peri-urban and rural settings, malaria transmitted by urban *An. stephensi* might divert resources to urban settings at the expense of rural settings, where low health system capacity and longer distances to health services means the risk of dying from malaria is more likely.

Conclusions

Anopheles stephensi, an invasive malaria vector in Africa, has been described as a potential threat to malaria control and elimination in Africa. First detected in 2016 in Ethiopia, *An. stephensi* now appears to be widespread, including in major urban and peri-urban areas, and remote rural areas along major transportation routes. Blood meal analysis showed that *An. stephensi* in Ethiopia were highly zoophagic, yet *P. vivax* sporozoite rates were higher than in the primary malaria vectors in Ethiopia, *An. arabiensis* and *An. pharoensis*, indicating potential to cause increases in malaria in urban areas. As vector control measures are considered, high levels of resistance to many of the insecticides used on ITNs and for IRS may render these interventions less effective, and therefore alternative interventions, such as new types of nets (PBO and bi-treated), and larviciding, may need to be considered.

Abbreviations

CDC: Centers for Disease Control and Prevention

CS: Circumsporozoite

DNA: Deoxyribonucelic acid

ELISA: Enzyme-linked immunosorbent assay

ITN: Insecticide treated bed nets

IRS: Indoor residual spraying

NMEP: National Malaria Elimination Program

PCR: Polymerase chain reaction

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the Institutional Review Board of Jigjiga University. It was also determined to be non-human subject research by the CDC Center for Global Health.

Consent for publication

Not applicable

Availability of data and material

All data generated are included in this manuscript and supplementary files.

Competing interests

The authors declare that they have no competing interests.

Funding

The funding for this work was provided by the U.S. President's Malaria Initiative.

Authors' contributions

MB, SI, and DD designed the field collection protocols; MB, PM, SI, DD, FG, EG, SC, HT, MM, and MY oversaw the collection of data; MB, GY, EA, DG, SY, AW, AG, EE, and TA collected the field data; DY conducted the laboratory work; SI, SZ, and MB analyzed and interpreted the data; MB, SI, and SZ wrote the first draft of the manuscript. DD, MY, and GY provided a critical revision of the manuscript. All authors have read and approve the content of the submitted manuscript.

Acknowledgements

The technicians who participated in mosquito collections and laboratory analysis are warmly thanked. We also thank community members who allowed collections to be made in their communities and homes. Jimma University Tropical and Infectious Diseases Research Center staff participating in ELISA work are also warmly thanked.

Authors' information

1 Abt Associates, PMI VectorLink Ethiopia Project, Addis Ababa, Ethiopia.

2 Dire Dawa University, Dire Dawa, Ethiopia.

3 Jigjiga University, Jigjiga, Ethiopia.

4 Addis Ababa University, Addis Ababa, Ethiopia

5 Armauer Hansen Research Institute, Addis Ababa Ehtiopia

6 Jimma University, Jimma, Ethiopia

7 US President's Malaria Initiative (PMI), Addis Ababa, Ethiopia.

8 United States Agency for International Development (USAID), Addis Ababa, Ethiopia.

9 Malaria Branch, Division of Parasitic Diseases and Malaria, Center for Global Health, Centers for Disease Control and Prevention, Atlanta, GA, USA.

10 US President's Malaria Initiative, USAID, Washington, DC, USA.

11 Abt Associates, PMI VectorLink Project, Rockville, MD, USA.

12 Entomology Branch Division of Parasitic Diseases and Malaria, Center for Global Health, Centers for Disease Control and Prevention, Atlanta, GA, USA.

Disclaimer

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention or the U.S. President's Malaria Initiative.

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Tables

Table 1

Sites sampled for larval and adult *Anopheles stephensi* in 2018-2020

Site	GPS coordinate	*Anopheles stephensi* present					
		2018		2019		2020	
		larval collections	adult collections	larval collections	adult collections	larval collections	adult collections
Assosa	10.062880, 34.543805			-			
Awash Sebat Kilo	8.988937, 40.160936	+	+	+	+	urban (+)/rural (+)	+
Bahirdar	11.591264, 37.381047			-			
Bati	11.191347, 40.014825	+	+			rural (+)	
Degehabur	8.223978, 43.558388	+	+			rural (+)	
Dire Dawa	9.602669, 41.840532	+	+	+	+	urban (+)/rural (+)	+
Erer Gota	9.555372, 41.384327	+	+				
Gambela	8.247653, 34.594831			-			
Gewane	10.157669, 40.660508	+	+	+		rural (+)	
Godey	5.952589, 43.556624	+	+			urban (+)/rural (+)	
Hawassa	7.053381, 38.489377			-			
Jigjiga	9.353974, 42.795313	+	+				
Jimma	7.669907, 36.837115			-			
Kebridehar	6.734321, 44.276404	+	+		+	rural (+)	+
Meki	8.152866, 38.823858			+		urban (+)/rural (-)	
Metehara	8.901551, 39.917774			+	+	urban (+)/rural (+)	+
Negelle-Borena	5.336451, 39.575286			-			
Semera	11.792397, 41.010032	+	+	+		rural (+)	
Shire	14.101822, 38.28188			-			
Yabello	4.893769, 38.097239			-			
Zeway	7.924096, 38.719499			+		urban (+)/rural (-)	

Collections that found *An. stephensi* are designated with a "+" and collections that were performed, but that did not find *An. stephensi* are designated with a "-"

Table 2

Larvae of *Anopheles stephensi* and other *Anopheles* species collected from various habitat types in selected urban sites in Ethiopia, August-December 2019

Urban site	Larval habitat type	Total *Anopheles* larvae collected	*An. stephensi*	*An. gambiae s.l.*	*An. rhodesiensis*	*An. cinereus*
Negelle-Borena	Water containers	55	0	13	0	0
	Water tanks	66	0	11	0	1
	Stagnant water pools	211	0	132	0	0
Yabello	Water tanks	39	0	13	0	0
	Stagnant water pools	55	0	23	0	0
	Cement water reservoirs	194	0	90	15	0
Jimma	Rain pools and puddles	378	0	148	0	0
Gambela	Rain pools and puddles	143	0	61	0	0
Assosa	Discarded tires	150	0	86	0	0
	Rain pools and puddles	1618	0	1266	0	0
	Natural habitats	710	0	531	0	0
Bahirdar	Tires	1681	0	1213	0	0
	Stagnant water pools	807	0	294	0	0
Meki	Tires	45	24	0	0	0
	Concrete water container	68	43	20	0	0
	Water tanks	36	19	10	0	0
	Discarded buckets	2	0	1	0	0
Zeway	Tires	24	14	0	0	0
	Water drums	1	0	0	0	0
	Concrete water containers	12	3	5	0	0
Hawassa	Water drums	7	0	5	0	0
	Concrete water containers	6	0	4	0	0
	Waste bin	12	0	9	0	0
	Plastic bucket	4	0	4	0	0
Shire	Tires	14	0	14	0	0
	Rain pools and puddles	2327	0	990	0	0
	Natural habitats	208	0	130	0	0
Metehara	Water tanks	1075	322	0	0	0

Table 3

Larval survey results of *Anopheles stephensi* and *Aedes* larvae in kebeles within 20km of urban sites in Ethiopia where *An. stephensi* had been found previously, 2020.

Nearest town	Number of visited kebeles	Number of kebeles positive for *An. stephensi*	Number of potential larval sites inspected	Number of larval sites positive for *An. stephensi* (%)	Number of larval sites positive for *Aedes* (%)	Number of *Anopheles stephensi* larval sites that also contained *Aedes* larvae (%)
Awash	1	1	3	1 (33)	1 (33)	1 (100)
Bati	7	3	165	6 (4)	42 (25)	4 (67)
Degehabur	6	2	32	7 (22)	7 (22)	2 (29)
Dire Dawa	7	2	17	2 (12)	8 (47)	2 (100)
Gewane	4	3	127	10 (8)	24 (19)	7 (70)
Godey	6	1	24	1 (4)	6 (25)	0
Kebridehar	8	6	40	13 (33)	6 (15)	0
Meki	5	0	17	0	0	0
Metehara	3	1	12	1 (8)	2 (17)	0
Semera	5	2	136	3 (2)	22 (16)	2 (67)
Zeway	3	0	16	0	0	0
TOTAL	55	21	589	44 (7)	118 (20)	18 (40)

Table 4

Anopheles stephensi collected in four longitudinal monitoring sites in Ethiopia in 2019 using different sampling methods. Sampling methods used in each site each month included: Human landing collections (HLC; 6 indoor and 6 outdoor collection nights), pyrethrum spray catches (PSC; 20 houses), CDC light traps (12 indoors and 12 outdoors), manual aspiration from animal shelters (2-20 collections per site), black resting boxes (6 nights), and animal baited tent traps (each for 3 nights).

Month 2019	Dire Dawa							Kebridehar							Awash S	
	PSC	HLC	CDC light trap	Hand Collection	Black resting box	Cattle-baited Tent Trap	Total	PSC	HLC	CDC light trap	Hand Collection	Black resting box	Cattle-baited Tent Trap	Total	PSC	HLC
June	0	0	0	ND	0	ND	0	4	1	3	ND	0	ND	8	ND	ND
July	3	0	0	18	0	ND	21	1	0	0	0	0	ND	1	ND	ND
August	1	0	0	127	16	16	160	2	0	0	5	0	6	13	0	4
September	0	0	3	24	82	9	118	4	0	0	19	0	18	41	0	2
October	0	0	1	26	56	9	92	1	4	0	79	0	29	113	2	1
November	0	0	0	5	2	6	13	3	0	0	46	0	29	78	0	0
December	0	0	0	5	3	0	8	14	0	0	63	0	37	114	1	0
Total	4	0	4	205	159	40	412	29	5	3	212	0	119	368	3	7

Table 5

Susceptibility test results of *Anopheles stephensi* in diagnostic and synergist assays

Type of assay	Insecticide class	Insecticide	Concentration	Percentage mortality (number tested)							
				2018		2019					2020
				Dire Dawa	Kebridehar	Dire Dawa	Kebridehar	Gewane	Semera	Awash Sebat Kilo	Awas Seba Kilo
Diagnostic dose	Pyrethroids	permethrin	0.75%	79 (100)	78 (100)	86 (100)	76 (100)	68 (100)	67 (100)	39 (100)	43 (100)
		deltamethrin	0.05%	54 (100)	80 (100)	64 (100)	74 (100)	31 (100)	37 (100)	68 (100)	15 (100)
		alpha-cypermethrin	0.05%	82 (100)	80 (100)	30 (100)	64 (100)	20 (100)	51 (100)	69 (100)	10 (100)
	Carbamates	bendiocarb	0.05%	19 (100)	73 (100)	4 (100)	17 (100)	60 (100)	57 (100)	5 (100)	13 (100)
		propoxur	0.10%	77 (100)	68 (100)	59 (100)	38 (100)	77 (100)	99 (100)	73 (100)	19 (100)
	Organophosphates	pirimiphos-methyl	0.25%	100 (100)	100 (100)	27 (100)	49 (100)	92 (100)	99 (100)	93 (100)	1 (100)
Synergist assays	Pyrethroids	permethrin	0.75%		69 (75)	85 (75)					70 (75)
		permethrin + PBO	0.75% / 4%		100 (75)	100 (75)					100 (75)
		deltamethrin	0.05%	45 (75)	49 (75)	67 (75)				61 (75)	21 (75)
		deltamethrin + PBO	0.05% / 4%	96 (75)	100 (75)	97 (75)				100 (100)	100 (75)
		alpha-cypermethrin	0.05%			83 (75)					31 (75)
		alpha-cypermethrin + PBO	0.05% / 4%			100 (75)					93 (75)

Table 6

Anopheles stephensi **lethal dose (LD)** LD_{50} **and** LD_{95} **values, with confidence intervals after exposure to temephos concentrations**

Site	LD_{50} (95%CI)	LD_{95} (95%CI)
Dire Dawa	0.105 (0.099-0.109)	0.118 (0.114-0.113)
Kebridehar	0.019 (0.015-0.027)	0.031 (0.024-0.122)
Meki	0.012 (0.011-0.013)	0.025 (0.021-0.032)

Figures

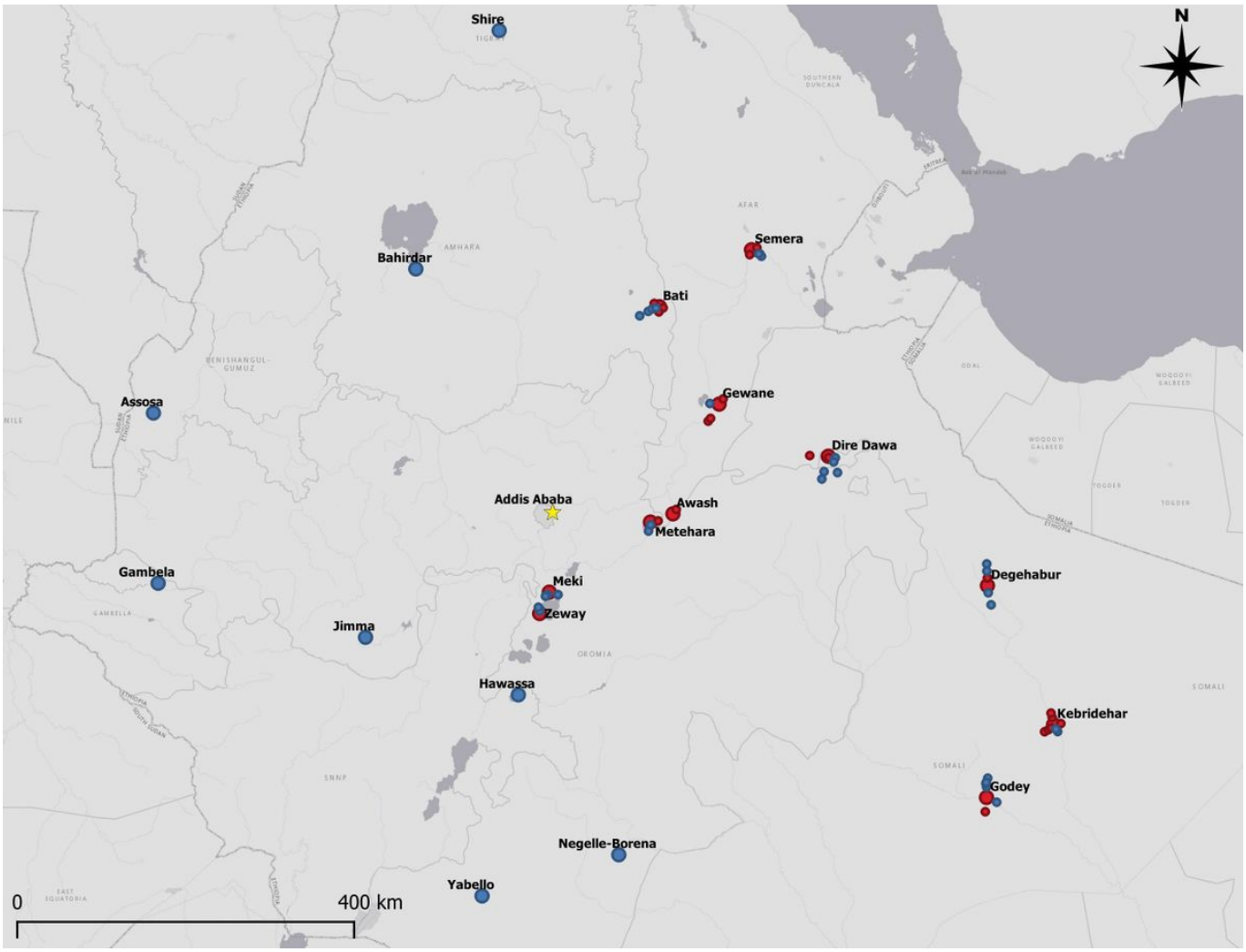

Figure 1

Sites positive (red) and negative (blue) for Anopheles stephensi in 2019 and 2020 Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

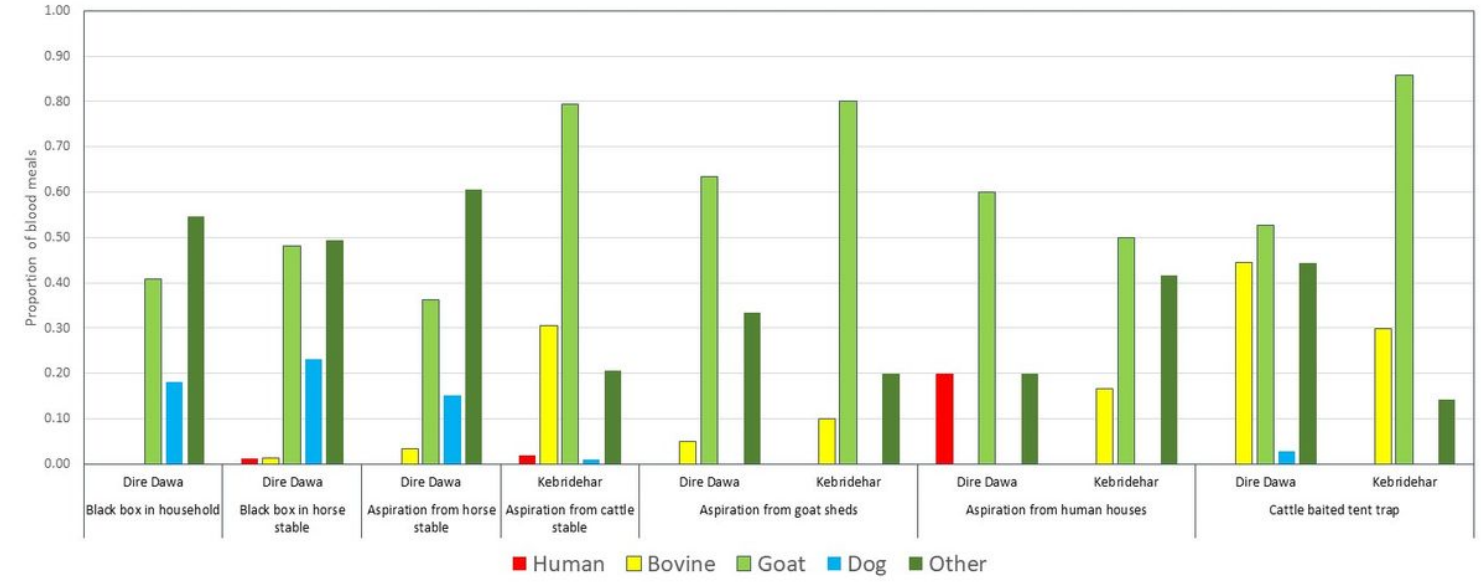

Figure 2

Identification of blood meal sources in adult Anopheles stephensi (2019) collected using different methods in Dire Dawa and Kebridehar, Ethiopia, 2019.

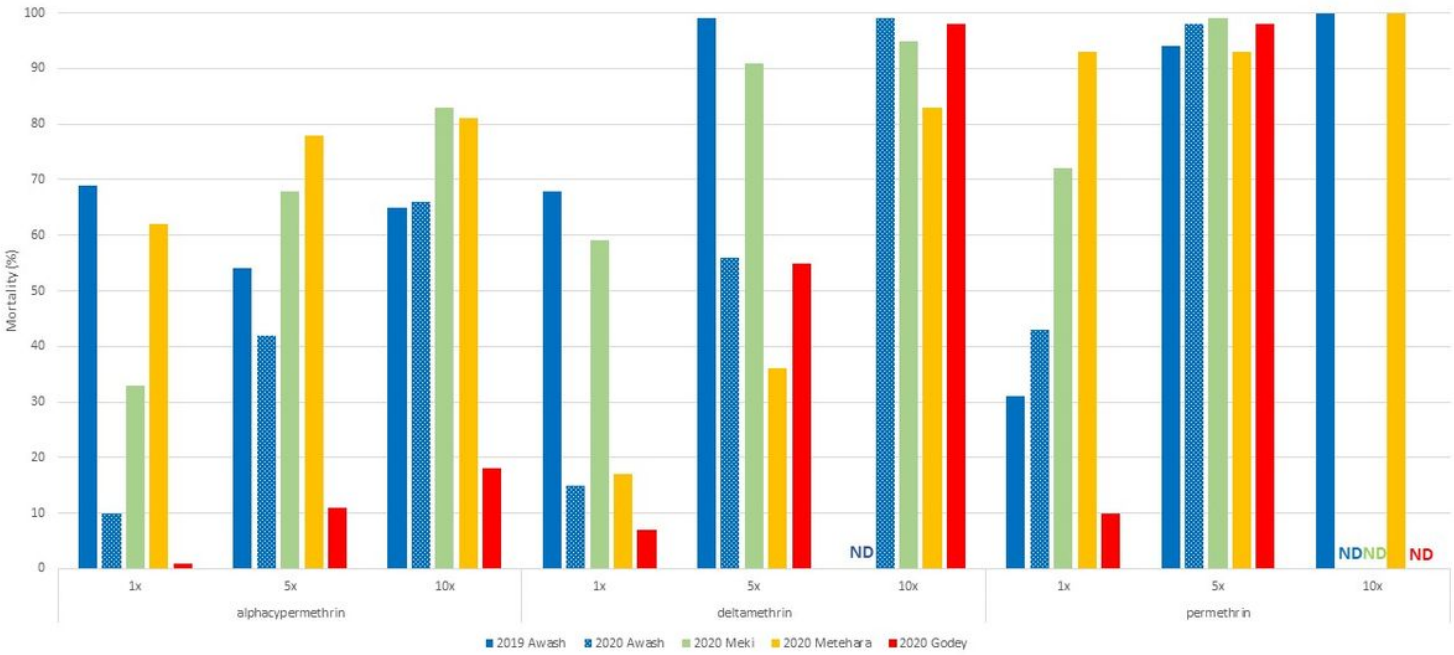

Figure 3

Intensity of resistance to pyrethroids in Anopheles stephensi in 2019 (Awash Sebat Kilo, designated Awash) and 2020 (Awash Sebat Kilo, Meki, Metehara, and Godey), Ethiopia. Tests were not done (ND) if susceptibility (>98%) was attained with a lower dose.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MASTER2020larvalsampling4.7.xlsx