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Elevated *Plasmodium* Sporozoite Infection Rates in Primary and Secondary Malaria Vectors in *Anopheles stephensi*-Infested Areas of Ethiopia

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Simple summary: Understanding sporozoite infection rates and blood meal sources in mosquito vectors is crucial for assessing the role of local vector species in malaria transmission. In this study, adult mosquitoes were collected and analysed to determine their blood meal sources and *Plasmodium* infection status. The study documented the presence of multiple efficient malaria vectors, including the recently introduced invasive vector, *Anopheles stephensi*. Both primary and secondary vectors exhibited a high *Plasmodium* infection rate, underscoring the importance of addressing secondary vectors in vector surveillance and control programs. Although *An. stephensi* showed no detectable infections, its presence adds complexity to malaria control efforts. This study highlights the need for continuous monitoring to elucidate the evolving role of different mosquito species in malaria transmission and to enhance control strategies.

Abstract: Evaluating blood meal sources and sporozoite infection rates of mosquito vectors species is important to determine their role in malaria transmission. The study assessed these parameters among malaria vectors in an area recently invaded by *Anopheles stephensi*. Adult mosquito surveys were conducted in Hawassa, southern Ethiopia between January 2023 and April 2023. Three adult mosquito collection tools were utilized: BG-Pro Traps, CDC Light Traps, and Prokopack Aspirator. Blood meal sources and sporozoite infection rates were determined using qPCR. Overall, 738 female *Anopheles* mosquitoes were collected, composed of *An. arabiensis* (72.9%), *An. pharoensis* (13.4%), *An. stephensi* (7.5%), and *An. coustani* (6.2%). The Human Blood Index (HBI) was 23.3% for *An. arabiensis*, 43.8% for *An. pharoensis*, 8.3% for *An. stephensi* and 25.0% for *An. coustani*. Of the mosquitoes tested, 8% of *An. arabiensis* and 4.7% of *An. pharoensis* were *Plasmodium*-positive, while *An. stephensi* and *An. coustani* showed no infection. The study documented multiple efficient malaria vectors, with high *Plasmodium* infection rates in both primary and secondary malaria vectors, highlighting the importance of secondary vectors in malaria transmission and the need to target them in malaria vector surveillance and control efforts. The coexistence of *An. stephensi* alongside efficient native vectors complicates malaria control strategies, necessitating continuous monitoring to better understand their evolving roles in malaria transmission.

Keywords: *Anopheles stephensi*; primary *vector*; secondary vector; sporozoite; blood-meal; Hawassa; Ethiopia

1. Introduction

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Understanding vector dynamics in disease transmission is crucial for designing effective vector control interventions against malaria. In Ethiopia, about 47 *Anopheles* species have been documented [1,2]. The primary vector of malaria is the *Anopheles* arabiensis, while *An. pharoensis, An. funestus, An. coustani complex*, and *An. nili* are secondary vectors [2]. Recently, the Asian malaria vector *Anopheles stephensi*, a competent vector of both *P. falciparum* and *P. vivax*, invaded East African region [3–5], complicating the existing malaria vectorial system.

Different malaria vector species exhibit varying blood meal preferences, influenced by host availability. Some are anthropophilic (preferring humans), others are zoophilic (preferring animals), or opportunistic (preferring both) [6–11]. These feeding preferences play a critical role in malaria transmission dynamics. Anthropophilic vectors present the highest risk because of their tendency to frequently feed on humans. Both *An. arabiensis* and *An. pharoensis* are considered an opportunistic feeder in Ethiopia [12–15]. *Anopheles stephensi*, on the other hand, is predominantly documented with zoophilic affinity [16,17].

In Africa, malaria transmission is facilitated by multiple *Anopheles* species, each exhibiting different vectorial capacities and behaviours [6]. In recent years, changes in vector dynamics have been documented in south and east Africa. For instance, Mustapha et al. [2021] indicated that secondary vectors may contribute substantially to malaria transmission because of their high densities and *Plasmodium*-positivity rate in Kenya [18]. This informs that in settings with multiple malaria vectors, continuous monitoring of their vectorial role is essential to understand the role of diverse vector species in malaria transmission and design control measures accordingly.

Hawassa City, the largest city in southern Ethiopia, is endemic to malaria with both *P. falciparum* and *P. vivax* prevalent [19,20]. The city has recently experienced an increase in malaria cases and incidence to epidemic level. Multiple malaria vectors have been reported in the area including *An. stephensi, An. arabiensis, An. pharoensis,* and *An. coustani* [19]. However, the role of diverse vector species in local malaria transmission has not yet been defined.

This study aims to identify the blood meal sources and sporozoite infection rates of *Anopheles* mosquitoes in Hawassa City, Ethiopia. Better understanding of vectorial characteristics helps guide the development of comprehensive control strategies and prevent recurrent outbreaks.

2. Methods

2.1. Study Design and Period

A targeted entomological survey was conducted from January 2023 to April 2023 in Hawassa City, 275 km from the Capital Addis Ababa in Southern Ethiopia (Figure 1). The Study setting was described elsewhere [19]. For vector surveillance, malarious villages (*kebeles*) were selected based on the report of the Hawassa City's Health Department [20].

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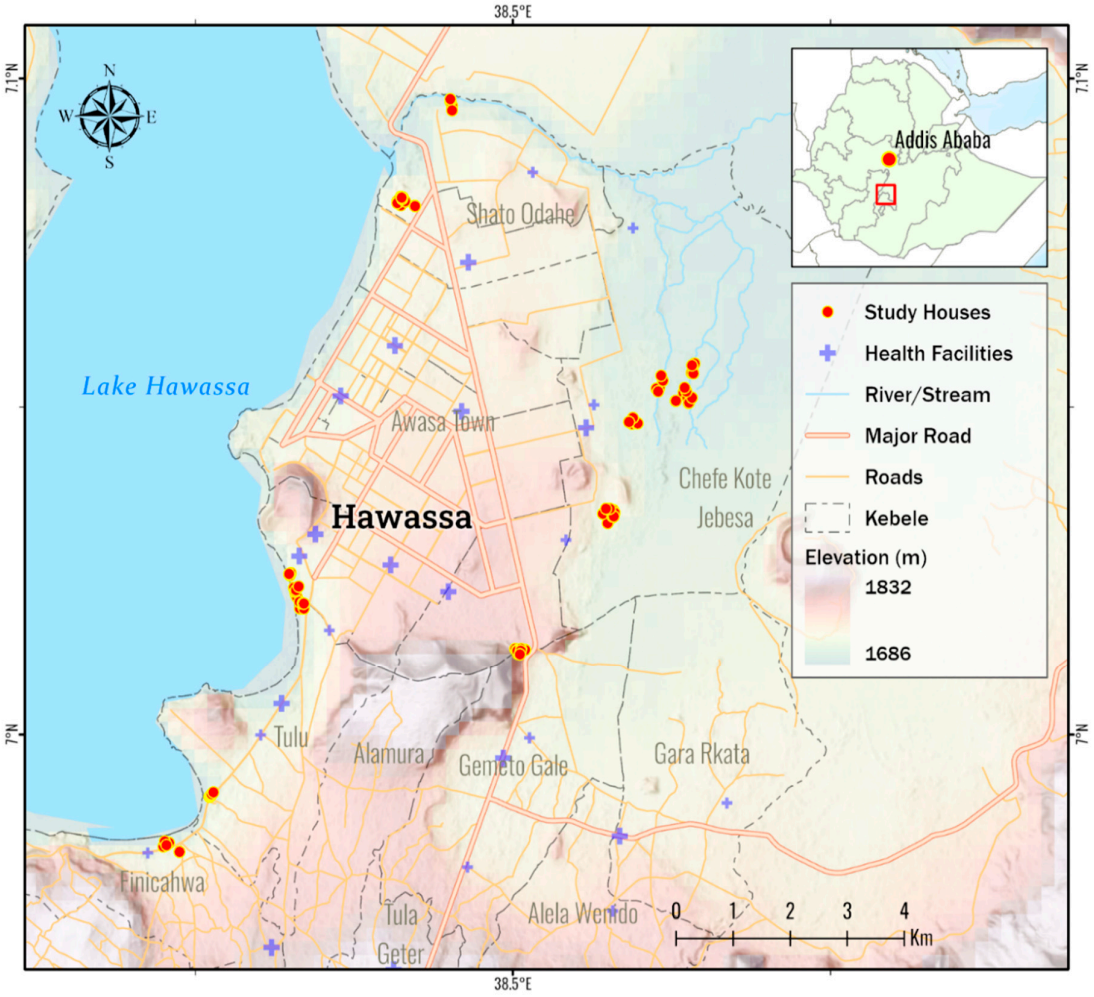

Figure 1. Map of the study setting, Hawassa City, Ethiopia.

2.2. Mosquito Survey

Three mosquito surveillance tools were employed to collect adult mosquitoes: CDC Light Traps (Model: John W. Hock CDC Light Trap 512, Gainesville, Florida, USA); Bioagents (BG-pro) Traps with attractant lure (Biogents AG, Regensburg, Germany); and Prokopack Aspirator (John W. Hock 1418, Gainesville, Florida, USA).

Indoor mosquito collections with CDC Light Trap and BG-Pro were conducted overnight from 18:00 to 06:00, with traps suspended 1.5 meters above the ground near sleeping areas. For the outdoor sampling, traps were set within 5-meter away from houses. A total of 180 and 120 trap-nights were set with BG-Pro and CDC Light traps, respectively. In addition, Prokopack Aspirator was used to sample indoor resting mosquitoes in the morning between 6:30 and 8:00 in 60 randomly selected houses.

2.3. Mosquito Specimen Processing

Collected mosquito specimens were brought to the Malaria Research Laboratory at Hawassa University for species identification. Live mosquitoes were euthanized using chloroform. The mosquitoes were then emptied into a petridish and sorted into culicines and anophelines. Culicines were counted, recorded, and discarded, while all *Anopheles* mosquitoes were further sorted out to species using a morphological key [21]. The abdominal status of each specimen was also assessed

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and categorized as unfed, fed, half-gravid, or gravid. Each female *Anopheles* mosquito was individually kept in labelled Eppendorf tubes for further molecular analysis.

2.4. Mosquito DNA Extraction

The head-thoracic part of each female adult mosquito was dissected for DNA extraction. DNA extraction was performed following the established automated DNA extraction protocol outlined by Zhong et al. [22]. Briefly, DNA extraction utilized the QIAamp 96 DNA QIAcube HT kit with a QIAcube HT 96 automated nucleic acid purification robot (Qiagen, Valencia, CA), following the manufacturer's protocol with minor modifications. Specifically, each sample was ground in a ZR Bashing Bead Lysis tube (2.0 mm beads, Zymo Research Corporation, Irvine, USA) and homogenized in 200 µl of lysis solution containing 20 µl of proteinase K (20 mg/ml) using the TissueLyser II system (Qiagen, Hilden, Germany) for 10 minutes at 30 Hz. The genomic DNA was eluted in a final volume of 100 µl for the head-thoracic portion and 200 µl for the abdominal portion. The DNA extracted from the head-thoracic portion was used for detecting *Plasmodium* sporozoites, while the DNA from the abdominal portion was used for species and bloodmeal identification [22].

2.5. Molecular Genotyping of Species, Bloodmeal, and Parasite Infections

For confirmation, *An. stephensi* and *An. arabiensis* was screened by PCR using a previously established protocol [19,23]. PCR reactions were carried out in a total volume of 17 µl, containing 1 µl of DNA template, 5 pmol of each primer, and 8.5 µl of DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, MA, USA). The thermocycling protocol involved an initial activation step of 3 min at 95 °C, followed by 35 amplification cycles of 30 s at 94 °C, 30 s at 55 °C, and 45 s at 72 °C, with a final extension step of 6 min at 72 °C. Other *Anopheles* species were identified through DNA sequencing of the ITS2 region of nuclear ribosomal DNA, following the methods described by Zhong et al. [22].

Mosquito blood meal sources (humans, cows, pigs, goats, and dogs) were examined using qPCR using previously established protocols [23–25]. *Plasmodium* infections (*Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*) in mosquitoes were examined by qPCR detection using established protocols [26–28]. Multiplexed qPCR was performed on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Carlsbad, CA) in a final volume of 12 µl, including 2 µl of sample DNA, 6 µl of PerfeCTa qPCR ToughMix, Low ROX Master Mix (2X) (Quantabio, Beverly, MA), 0.5 µl of each probe (2 µM), and 0.4 µl of each forward primer (10 µM) and reverse primer (10 µM). The temperature profile involved a hold stage at 50 °C for 2 min and 95 °C for 2 min, followed by 45 cycles of PCR amplification at 95 °C for 3 s and 60 °C for 30 s.

2.6. Data Analysis

Mosquito density was calculated as the mean number of mosquitoes caught per trap – night for each trap type. *Anopheles* species density difference across traps was compared using independent Samples Kruskal-Wallis Test. *Plasmodium* sporozoite infection rate was calculated as the proportion of mosquitoes infected with *Plasmodium* against those tested. Entomological inoculation rates (EIR) was estimated by multiplying the sporozoite rate by man-biting rate; Man-biting rates were derived from traps catches (i.e., density divided by a conversion factor 1.605 [29]). The human blood index (HBI) and bovine blood index (BBI) were calculated as the proportion of fed mosquitoes that fed on human and bovine blood meals, respectively [8,30,31]. Mixed blood meals (human + bovine) were included in the counts for both HBI and BBI. All data was analysed using Microsoft Excel (Version 2016, Microsoft Corp, USA) and IBM SPSS version 25.0 (SPSS Inc., Chicago, IL, USA).

2.7. Ethical Considerations

The study protocol was approved by the Institutional Review Board (IRB) of the College of Medicine and Health Sciences, Hawassa University. Mosquito collections from each household were conducted after obtaining verbal consent from the household head.

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3. Results

3.1. Anopheles Density

A total of 738 female *Anopheles* mosquitoes were collected during the study period. *Anopheles arabiensis* was the predominant species, accounting for 72.9% (n=538) of the collections, followed by *An. pharoensis*, 13.4% (n=99), *An. stephensi*, 7.5% (n=55), and *An. coustani* 6.2% (n=46). A higher density of *An. arabiensis* was recorded in the BG-Pro Trap collection compared to other trapping techniques (df = 2; P = 0.004), while a higher density of *An. pharoensis* was observed in CDC Light Trap collection (P = 0.006). *Anopheles stephensi* was exclusively captured by the BG Pro Trap and the ProkoPack Aspirator in a comparable density (P = 0.254). In both the BG Pro and CDC LT collections, more *An. arabiensis* was collected indoors than outdoors. In contrast, for *An. pharoensis*, outdoor density exceeded indoor density by 1.5 times in the BG Pro collection and by 1.3 times in the CDC collection (Figure 2).

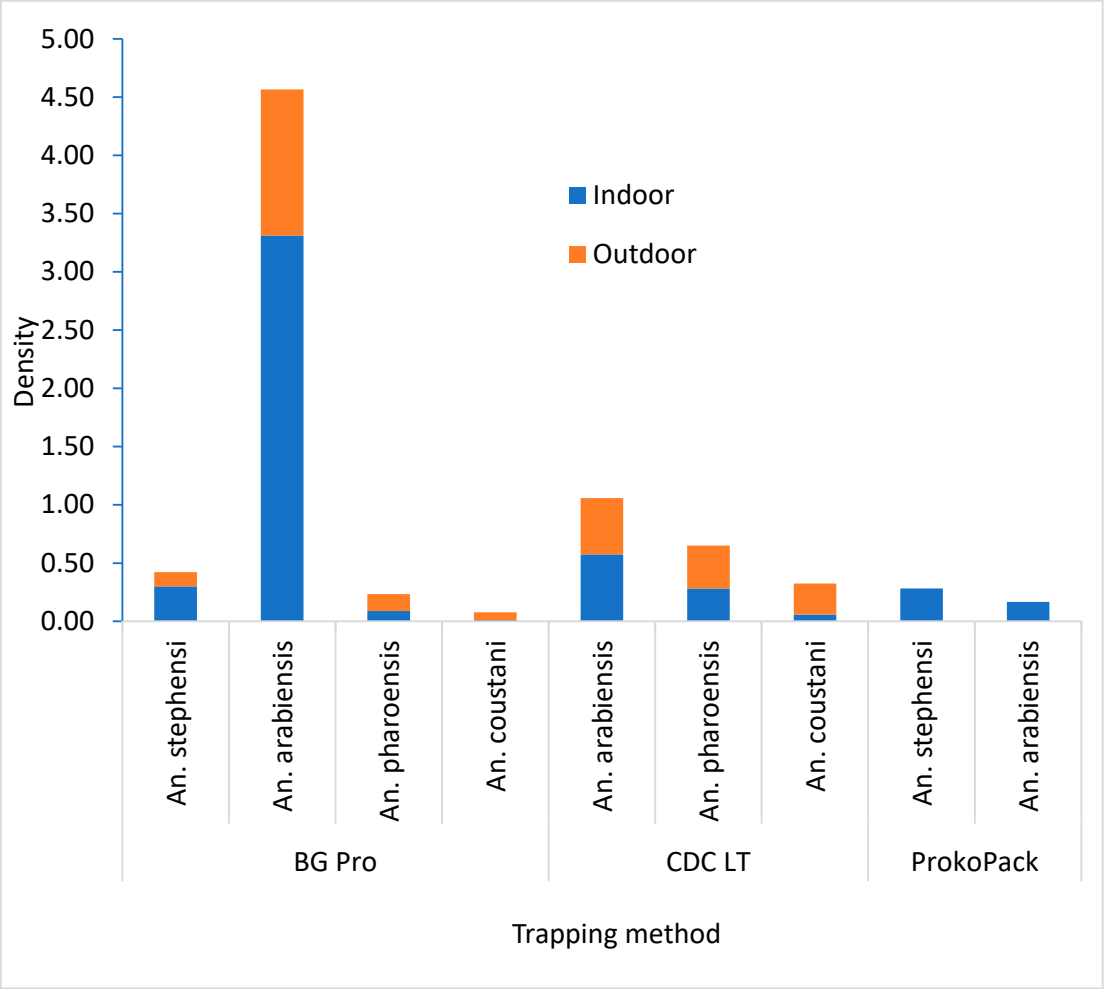

Figure 2. Density of *Anopheles* mosquitoes in various trapping devices in Hawassa, southern Ethiopia, 2023.

3.2. Blood Meal Source

A total of 456 *Anopheles* mosquitoes were tested for blood meal sources. Blood meals sources identified included human, cow, goat, and dog (Table 2). Some (37.5%) of the samples were unidentified.

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Anopheles arabiensis and *An. stephensi* had a lower Human Blood Index (HBI) value, 23.3 % and 8.3%, respectively. These species however demonstrated a higher Bovine Blood Index (BBI), 61.3% and 75.0%, respectively. *An. pharoensis* showed similar HBI (43.8%) and BBI (41.7%) (Figure 3).

Table 2. Blood meal sources of *Anopheles* species in Hawassa, southern Ethiopia.

Host	***An. arabiensis***	***An. pharoensis***	***An. stephensi***	***An. coustani***
	n (%)	**n (%)**	**n (%)**	**n (%)**
Human	35(10.6)	14(16.5)	1(3.3)	1(10.0)
Human + cow	18(5.4)	3(3.5)	1(3.3)	1(10.0)
Human + cow + goat	1(0.3)	2(2.3)	-	-
Human + goat	5(1.5)	2(2.4)	-	-
Cow	116 (35.0)	12(14.1)	16(53.3)	3(30.0)
Cow + goat	19(5.7)	3(3.5)	1(3.3)	-
Cow + goat + dog	1(0.3)	-	-	-
Dog	1(0.3)	-	-	-
Goat	11(3.3)	13(15.3)	3(10.0)	2(20.0)
Not detected	124(37.5)	36(42.4)	8(26.7)	3(30.0)
Total	**331**	**85**	**30**	**10**

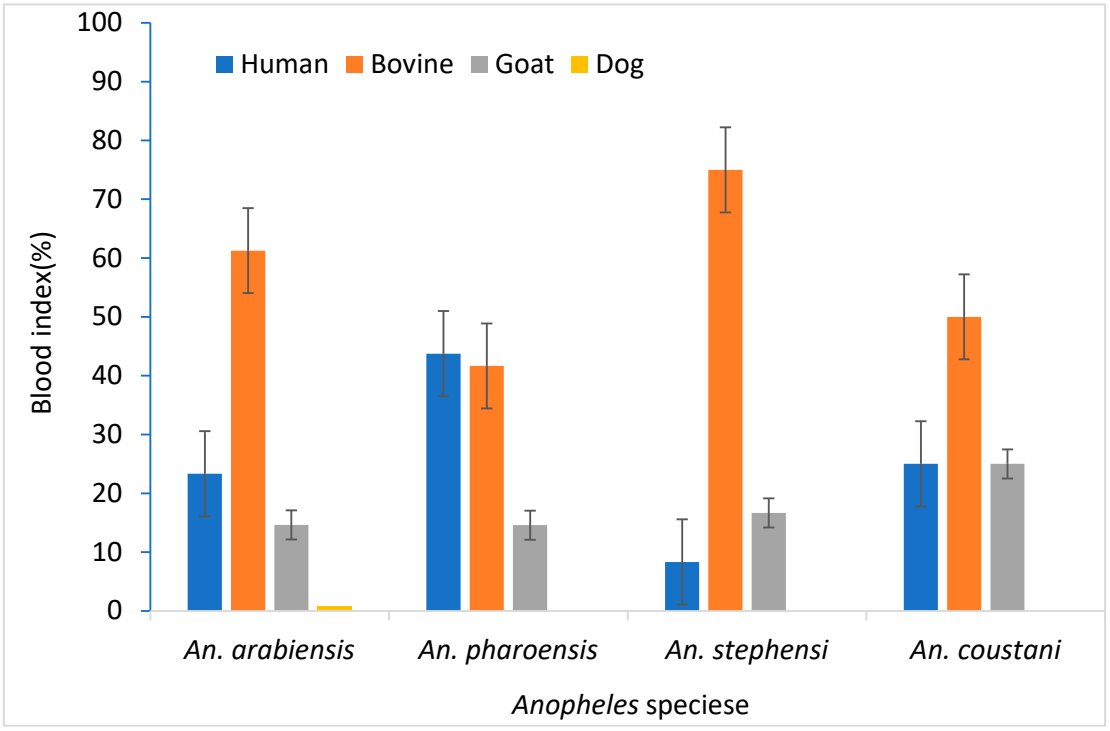

Figure 3. *Anopheles* species blood meal index in Hawassa, Southern Ethiopia, 2023.

3.3. Sporozoite Infection and Entomological Inoculation Rates

PCR results showed 4.0% of *Plasmodium falciparum* and 4.0% of *Plasmodium vivax* sporozoite infection in *An. arabiensis*. Likewise, 3.5% of *P. vivax* and 1.2% of *P. falciparum* was detected in *An. pharoensis*, while no infection was observed in both *An. stephensi* and *An. coustani* (Table 3).

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The EIR estimated for the study period showed that the study area received 4.50 and 0.25 *P. falciparum*-infective bites and 4.50 and 0.72 *P. vivax*-infective bites each night by *An. arabiensis* and *An. pharoensis*, respectively.

Table 3. *Plasmodium* sporozoite infection rates of *Anopheles* species, Hawassa, Ethiopia, 2023 .

***Anopheles* species**	**Number tested**	**Sporozoite positive n (%)**		**Parasite species**			
		Indoor collection	Outdoor collection	*Plasmodium* falciparum		*Plasmodium* vivax	
				Indoors	Outdoors	Indoors	Outdoors
An. arabiensis	331	13 (4.0)	13 (4.0)	7	6	6	7
An. pharoensis	85	3 (3.5)	1(1.2)	1	-	2	1
An. stephensi	30	0	-	0	-	0	-
An. coustani	11	0	-	-	-	-	-

4. Discussion

The present study highlighted an elevated *plasmodium* sporozoite infection rate in primary and secondary malaria vectors of Ethiopia as compared to previous reports. *Plasmodium* sporozoite infection rate of *An. arabiensis* reported was about seven times higher than in previous studies in central and southern Ethiopia [8,32–36]. Similarly, the observed sporozoite rate in *An. pharoensis* was about six-times higher than previously reported [32,33,36]. A recent study also documented the increase of malaria incidence in the study area [19], which might be attributed to the high sporozoite infection rate observed in this study in both primary and secondary vectors. This is mainly because the area is prone to malaria epidemics due to the presence of mosquito-breeding lake shorelines of Lake Hawassa coupled with swampy areas surrounding the city. The present findings suggest the need of inclusive interventions considering both primary and secondary vectors for successful control of malaria in the area. Current control measures predominantly target the primary vectors. However, it is noted that the secondary vectors such as *An. pharoensis and An. coustani*, could contribute to residual transmission [6]. This suggests that in regions with multiple efficient malaria vectors, the interventions targeted a primary vector alone may not effectively reduce malaria transmission as other vectors could keep augmenting the transmission. Additionally, potential vector shift may also occur if targeted intervention suppresses the primary vector population as noted by Msugupakulya *et al.* (2023) in East and Southern Africa [37].

Malaria transmission in Ethiopia is a complex and dynamic issue, with a substantial gap in understanding the bionomics and role of secondary vectors in disease transmission. *Anopheles pharoensis* was incriminated as the second most abundant malaria vector long time ago and exhibited the second-highest *Plasmodium* infection rate after *An. arabiensis* in this study, suggesting its significant role in local malaria transmission. Several studies also noted that *An. pharoensis* has consistently ranked second in abundance and sporozoite infection rate elsewhere in Ethiopia [32,33,35]. In another laboratory study, *An. pharoensis* demonstrated similar susceptibility with *An. arabiensis* to *plasmodium falciparum* infection [38]. Unfortunately, less attention has been given to the secondary vectors both in research and vector control interventions in Ethiopia. The study emphasizes the need for a comprehensive understanding of secondary vectors biology, ecology, behavior, vector competence, and response to the existing interventions. As the country embraces to malaria elimination, accurate information on the role of multiple vector species on malaria transmission is critical.

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The presence of *An. stephensi* could further posed a significant threat to malaria control efforts in Ethiopia [3]. As the species alarmingly spread to several areas in Ethiopia [4,19,39], control measures are still not well incorporated in routine vector control strategies. Few studies from eastern Ethiopia have linked the recent regional malaria outbreak to *An. stephensi* [40,41]. In this study, this species was occurred in sympatry with native vectors with low density in adult collections in southern Ethiopia. A recent study in the same study area reported high larval density of *An. stephensi* [19]. This indicates the species is well established in southern Ethiopia. Although no *An. stephensi* specimens tested positive for *Plasmodium* sporozoites in this study, probably due to small sample size due to absence of optimal vector surveillance tools for *An. stephensi* adult collection, this species can be of potential risk for malaria transmission [4,39]. Therefore, it is advisable to intensify to further develop efficient surveillance tools to collect a good sample size of adult *An. stephensi* specimens for testing, hence better defining its actual role in malaria transmission in the region. The lower HBI and *Plasmodium* infection rates observed in wild *An. stephensi* populations, both in this study and earlier ones from Ethiopia [4,39], are may be hypothesized to result from the mysorensis form of this species which has zoophilic behavior, spreading in Ethiopia (unpublished data). Future research should test these hypotheses in the invaded regions of Ethiopia.

In summary, the study documented the presence of multiple efficient malaria vectors, with *An. arabiensis* and *An. pharoensis* showing high *Plasmodium* infection rates, highlighting the importance of secondary vectors in malaria transmission and the need to address them in control efforts. The coexistence of *An. stephensi* alongside these primary and secondary vectors adds complexity to malaria control strategies in the area, necessitating continuous monitoring to understand the bionomics and evolving roles of mosquito vector species and species shift in malaria transmission.

Author contributions: DH and GY contributed to the conception and design of the study. DH, TA, AA, AE, TM, and CA organized and led the collection of specimens. DH analysed the data and drafted the manuscript. MCL and GZ were involved in data analysis. DZ, CW, JC, AL, NS, and KKK involved in laboratory analysis.GY, DY, and SK critically reviewed the manuscript for intellectual content. All authors read and approved the final manuscript.

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Data Availability Statement: The datasets are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The author reports no conflicts of interest in this work.

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