1 Lack of robust evidence for a *Wolbachia* infection in *Anopheles gambiae* from
2 Burkina Faso.

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23

24 **Abstract.**

25 The endosymbiont *Wolbachia* can have major effects on the reproductive fitness,
 26 and vectorial capacity of host insects and may provide new avenues to control
 27 mosquito borne pathogens. *Anopheles gambiae* s.l is the major vector of malaria in
 28 Africa but the use of *Wolbachia* in this species has been limited by challenges in
 29 establishing stable transinfected lines and uncertainty around native infections. High
 30 frequencies of infection of *Wolbachia* have been previously reported in *An. gambiae*
 31 collected from the Valle du Kou region of Burkina Faso in 2011 and 2014. Here we
 32 re-evaluated the occurrence of *Wolbachia* in natural samples, collected from Valle du
 33 Kou over a 12-year time span, and in addition, expanded sampling to other sites in
 34 Burkina Faso. Our results showed that, in contrast to earlier reports, *Wolbachia* is
 35 present at an extremely low prevalence in natural population of *An. gambiae*. From
 36 5,341 samples analysed only 29 were positive for *Wolbachia* by nested PCR
 37 representing 0.54% of prevalence. No positive samples were found with regular
 38 PCR. Phylogenetic analysis of 16S rRNA gene amplicons clustered across
 39 supergroup B, with some having similarity to sequences previously found in
 40 *Anopheles* from Burkina Faso. However, we cannot discount the possibility that the
 41 amplicon positive samples we detected were due to environmental contamination or
 42 were false positives. Regardless, the lack of a prominent native infection in *An.*
 43 *gambiae* s.l. is encouraging for applications utilising *Wolbachia* transinfected
 44 mosquitoes for malaria control.

45

46 **Keywords:** *Wolbachia*, *Anopheles gambiae*, wAnga, biocontrol, native infection,
 47 population suppression, population replacement.

49 Background

50 *Wolbachia* is an obligate intracellular bacterial symbiont, found in many insect
 51 species that, in recent years, has shown great potential use for use in vector-borne
 52 pathogen control. It is well known for its ability to manipulate host reproduction
 53 enabling it to spread into insect populations (Werren et al., 2008). Additionally,
 54 *Wolbachia* can inhibit the development of diverse pathogens (Hedges et al., 2008;
 55 Kambris et al., 2009; Moreira et al., 2009; Hughes et al., 2011; Kambris et al., 2010;
 56 Shaw et al., 2016) which makes *Wolbachia* an attractive agent for pathogen control.
 57 Some strains of *Wolbachia* protect insect hosts from viral infections (Chrostek et al.,
 58 2013; Hedges et al., 2008; Glaser et al., 2010) and the presence of *Wolbachia* in
 59 *Aedes aegypti* mosquitoes impair infections with dengue and other arboviruses
 60 (Walker et al., 2011; Moreira et al., 2009). Based on these findings, releases of
 61 *Wolbachia* infected male and female mosquitoes have been undertaken with the aim
 62 of spreading *Wolbachia*-mediated resistance to viruses in natural mosquito
 63 populations (Hoffmann et al., 2011) with initial clinical trials showing positive
 64 outcomes (Indriani et al., 2020; Utarini et al 2021). In addition to population
 65 replacement approaches, *Wolbachia* has also been exploited for population
 66 suppression control strategies whereby infected male are released to reduce
 67 mosquito numbers by inducing cytoplasmic incompatibility when mating with
 68 uninfected females (LAVEN, 1967; Zheng et al., 2019; Crawford et al., 2020)

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70 In *Anopheles* mosquitoes there are several reports indicating amplification of the
 71 *Wolbachia* 16S rRNA gene fragment by nested PCR. In general, these studies find
 72 *Wolbachia* PCR positive individuals at low frequency and infection prevalence in the
 73 population (Baldini et al., 2014; Shaw et al 2016; Ayala et al., 2019; Gomez et al.,

2017), however some studies reporting a negative correlation between amplicon positive mosquitoes and *Plasmodium* development in natural populations (Shaw et al., 2016; Gomez et al., 2017). Recently it has been shown that *Anopheles moucheti* and *An. demeilloni* possess high density infections with *Wolbachia* infections being observed in the germline, and complete genomes recovered (Jeffries et al., 2018; Walker et al., 2021; Quek et al., 2021). Significant efforts to establish artificially transinfected lines of *Anopheles* with *Wolbachia* have proved largely unsuccessful (Walker et al., 2011). Importantly, a stable line was established in *An. stephensi*, a vector of malaria in southern Asia, using *Wolbachia* from *Ae. albopictus* (wAlbB), which conferred resistance to *Plasmodium falciparum* infection (Bian et al., 2013). However, the *Wolbachia* infection induced fitness costs on the host, which would likely prevent establishment of the bacterium in mosquito populations (Joshi et al., 2014). Somatic, transient infections of the *Wolbachia* in *An. gambiae* were shown to significantly inhibit *P. falciparum* (Hughes et al., 2011), but the interference phenotype is variable (with other *Wolbachia* strain-parasite combinations (Hughes et al., 2012; 2014; Murdock et al., 2014). Interestingly, *Wolbachia* and other gut-associated microbes have negative associations (Hughes et al 2014; Rossi et al., 2015; Zink et al 2015), and these microbial interactions affect the biology of the host and transmission of the bacterium (Hughes et al., 2014), offering a possible reason for the lack of infection in some *Anopheles* species.

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Despite the widespread report of amplification of *Wolbachia* by PCR in *Anopheles*, there is conjecture in the literature if *Anopheles* mosquitoes are truly infected with *Wolbachia* (Chrostek & Gerth, 2019). Most of the evidence stems from nested PCR approaches, a technique that is highly sensitive. This has led to suggestions that

99 nested PCR may detect environmental *Wolbachia* DNA that the mosquito has
 100 encountered (Chrostek & Gerth, 2019). The low density and prevalence of infection
 101 and the phylogenetic diversity of strains reported indicates the infection is not stable
 102 in these associations or environmental DNA is being amplified. It is imperative to
 103 verify the prevalence of natural infections, and identify any native strains of
 104 *Wolbachia* in *Anopheles*, as these infections could impede population suppression or
 105 replacement control approaches exploiting transinfected lines. We previously
 106 reported high levels of natural infection in *An. gambiae* from Burkina Faso (Baldini et
 107 al., 2014; Shaw et al., 2016), but here we re-evaluate *An. gambiae* mosquitoes from
 108 these sites to examine the prevalence of infection in a wider geographical across a
 109 temporal sample set. Surprisingly we find low levels of amplicon positive mosquitoes,
 110 calling into question the findings from our earlier study.

111

Methods

Study area and mosquito collection

Anopheles gambiae s.l. mosquitoes were collected in seven villages during the rainy seasons of 2006, 2011, 2012, 2015, 2016 and 2018. These sites are located in the Sahelian zone (Kongoussi and Yilou) and Sudan-savanna zone (VK3, VK5, VK7, Soumousso and Tiefora) (Figure 1) of Burkina Faso. Adult mosquitoes were collected from the resting sites (inhabited houses, uninhabited houses, wood piles, clay pots) using mechanical aspirators or CDC light traps; males were also collected from mating swarms using an insect net (Diabaté et al., 2006). Adults were morphologically identified using the standard taxonomic key (Gillies & Coetzee, 1987, Gillies & de Meillon, 1968). Samples were stored at -20°C prior to molecular analyses.

Mosquitoes DNA extraction and molecular analysis.

DNA was extracted from 4953 *An gambiae* s.l. mosquitoes using the CTAB 2% extraction method. PCR was used to identify the species, as described previously (Santolamazza et al., 2008). To screen for *Wolbachia*, conventional (Werren & Windsor, 2000) or nested-PCR using *Wolbachia* specific primers was used to amplify the 16S rRNA gene (Shaw et al., 2016). Amplicon positive samples were repeated to confirm infection and the PCR products purified by filtration using NucleoFast ®96 PCR DNA purification plate. For Sanger sequencing Positive (DNA from *Culex* mosquitoes collected in the city of Bobo-Dioulasso) and negative controls were included to rule out PCR contamination. Samples were sequenced by GenoScreen.

In parallel to the 4953 samples we analyzed, a total of 388 samples, all collected as larvae either from VK7 (n=268) or from the Cascades district (n=120) were analysed at LSTM. These Samples were collected between October 2011 and September 2014 and included 292 samples that had been exposed to deltamethrin (199 survivors, 93 dead) (see Supplementary file S1). All were tested using the 16S-Wspecf/Wspecr primers (Werren et al., 2000).

Phylogenetic analysis

Phylogenetic analyses of *Wolbachia* samples were performed on the conserved 16S rRNA sequences. Accession numbers of the sequences obtained in the present study and used to construct the tree are listed in Table 1. Contigs obtained were cleaned and assembled *de novo* using Geneious v. 8.1.7 (Biomatters Ltd). All sequences were subjected to BLAST search tools in NCBI using Geneious and subsequently to pairwise sequence comparison (Altschup et al., 1990; Bao et al., 2014). The homologous sequences were retrieved for phylogenetic analysis based on BLAST results.

From this, we obtained a total of 70 sequences of 16S RNA from *Wolbachia* of supergroups A and B. This number also included 13 sequences that were reportedly isolated from *An. gambiae*. These 70 sequences were added to the 17 sequences obtained from this study, as well as an additional sequence from *Wolbachia* of *Culex* mosquitoes, sequenced as part of this study. All of these 88 sequences were then used as input into the program MAFFT (v7.455 Katoh and Standley, 2013), and aligned using default parameters. The resultant alignment was then manually processed to remove ambiguously aligned regions at the 5' and 3' ends, as well as any columns that contained undetermined nucleotides from sequencing. This

curated alignment was then used as input into the program IQTree (v1.6.1, Nguyen et al., 2015) to build a phylogenetic tree, utilising the DNA substitution model K2P+G4 (best-fit model as determined by the ModelFinder algorithm, Kalyaanamoorthy et al., 2017), with 1,000 non-parametric bootstrap replicates. The tree was then visualized and edited using FigTree v.1.4.4.

Results

No amplicon positive samples were found with the regular PCR but a total of 46 of the 4,953 amplicons were positive for *Wolbachia* using the more sensitive nested PCR representing a prevalence of 0.93%. The nested PCR was repeated for each of the initial 46 positive mosquitoes and 29 remained positive on the repeat PCR, representing a prevalence of 0.59% (29/4953). The inability to confirm 17 individuals in a repeated nested-PCR suggested the template is at the limits of detection in these samples.

Wolbachia was only detected in samples from two of the six years; in 2006, *Wolbachia* prevalence was 5.33 % (n= 507) and 0.08 % (n=2490) in 2018 (Table 1). Regarding the spatial distribution, all positive specimens were found in just three of the seven villages (VK3, VK7 and Soumoussou), all located in the Sudan savanna zone in the western region of Burkina Faso. The prevalence of positive samples was low with 0.31% (2/638) in VK3, 0.36% (6/1376) in VK7 and 1.87%: (21/1150) in Soumoussou. A significantly higher proportion of *An. gambiae* s.s were amplicon positive for *Wolbachia* than *An. coluzzii* ($\chi^2 = 23.493$, df = 1, p < 0.0001; *An. gambiae* 2.08% (24/1150); *An. coluzzii* 0.53% (22/4151)

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185 Concerning the 388 samples analyzed in LSTM from VK7 and Tiefora no positive
186 amplicon was found in any sample. However, positives bands were observed in
187 positive control using the same protocol.

188

189 **Phylogenetic Analysis of *Wolbachia***

190 Phylogenetic analysis of the 16S rRNA gene sequences used in this study (70
191 published sequences, 17 from this study, one positive control from *Wolbachia* of
192 *Culex pipiens*) resulted in a curated alignment of 317 nucleotides in length. A
193 midpoint-rooted tree showed that all sequences obtained from this study clustered
194 with supergroup B *Wolbachia*. From this study, 12 sequences of *An. gambiae* and
195 *An. coluzzii* were noted to cluster into a weakly supported clade (Figure 2) alongside
196 wAlbB, and six previously published sequences collected from *An. gambiae* in
197 Burkina Faso and Guinea. The remaining six sequences obtained from this study
198 were observed to be distributed throughout supergoup B, with none in supergoup A.
199 Contrasting with the sequences from this study, previously published sequences
200 from *An. gambiae* were observed to be distributed throughout both supergroups A
201 and B (Figure 2).

202

203 When the alignment was reduced to look at the 18 sequences from this study, a total
204 of six variant sites were observed, with five of these sites being unique to one
205 sequence (Supplementary file S2, sample Sawadogo-15). Expanding this alignment
206 to include all sequences from *Anopheles* mosquitoes, as well as *Wolbachia* from *A.*
207 *albopictus*, a total of 31 variant sites were observed. The majority of these variant

sites were observed to occur within seven of the published *Wolbachia* sequences, all of which cluster within supergroup A of the phylogenetic tree.

Discussion

Despite being common among *Culex* and some *Aedes* mosquitoes (Carvajal et al., 2019), *Wolbachia* infections are relatively rare in most *Anopheles* species. While there are emerging reports suggesting that some *Anopheles* possess low prevalence infections (Baldini et al., 2014; Shaw et al., 2016; Ayala et al., 2019; Gomez et al., 2017), these studies use nested PCR which is a highly sensitive technique that could amplify environmental DNA. As such the veracity of these studies has been questioned (Chrostek and Gerth 2019). In contrast, a recent study demonstrated that *An. moucheti* and *An. demeilloni* possessed high-density maternally transmitted *Wolbachia* strains (Walker et al., 2021). Further genomic analysis of these strains indicated similar metabolic pathways to other *Wolbachia* strain and a close relationship to *Wolbachia* strains found in *Drosophila simulans* and *D. mauritiana* (Quek et al., 2022). Our expanded survey of *Wolbachia* from *An. gambiae* collected in Burkina Faso found an extremely low prevalence of *Wolbachia* using the nested-PCR screening approach.

The intensity of infection in most *Anopheles* is far lower than found in other insects. Analysis of 4,953 samples collected over a twelve-year period in Burkina Faso found only 0.59% (29/4953) were amplicon positive for *Wolbachia*. This contrasts with an infection rate of 46% (275/602) in *An. coluzzii* in 2014 in the same area (Shaw et al., 2016) and varying spatially and temporally with samples from Mali with a minimal prevalence of 45% in Dangassa in 2015 reaching 95% (38/40) in *An. gambiae* sl in Kenieroba in 2016 (Gomez et al., 2017). A separate screening conducted in parallel

at the Liverpool School of Tropical Medicine, to detect *Wolbachia* in *An. gambiae* sl. survivors of pyrethroid exposure collected in 2011 and 2012 from the same geographic region, failed to detect any *Wolbachia* samples in 348 individuals screened. Corroborating our findings, more recent studies using amplicon sequencing of the 16S rRNA gene have found minimal evidence for *Wolbachia* infection of *An. gambiae* in Burkina Faso. The relative abundance of the *Wolbachia* in the *An. gambiae* microbiome was 0.00002% (Zoure et al., 2019), while amplicon sequencing of nested-PCR positive individuals only found 42 *Wolbachia* reads constituting 0.04% relative abundance of the microbiome (Straub et al., 2020). Phylogenetic analysis indicated low levels of diversity within the 18 sequences obtained in this study, with sequences from both *An. gambiae* and *An. coluzzii* showing few variants. Furthermore, a total of 12 of these sequences appeared identical to that found from wAlbB. Interestingly, several sequences originally isolated from *An. gambiae* also appeared closely related to this group. One of the 18 sequences identified in this study did show significant divergence, clustering with sequences found within other insects.

While some of our amplicons were Sanger sequenced and had similarity to *Wolbachia* we cannot discount the possibility these were false positives. While we did not explicitly determine the specificity rate of this nested PCR, presuming it had a specificity rate of 99%, we would expect on average 49 false positive PCRs. These figures are in line with our 29 positive samples. Similarly, these amplicon positives samples, several of which appear to be on the limits of detection, could have been due to amplification of amplified environmental DNA encoding for the *wsp* gene. For example, it is known that microbial DNA can persist within soil for years (Nielsen et

al., 2007). Finally, it is possible that the signal was due to *Wolbachia* bacteria that were transiently associated with the mosquito. This may be facilitated by ectoparasitic mites or midges, infection with endoparasitic nematodes, cohabitation with other arthropods hosting these bacteria, or acquired through the nectar feeding in plants (Chrostek et al 2017). Interestingly, while our current study shows that *Wolbachia* is present at a very low prevalence in *An. gambiae*, we saw more amplicon positive samples from villages (Soumouso (6.34%) and VK7 (3.41%)) where it had been detected previously in 2006 (Baldini et al., 2014; Shaw et al., 2016). This could possible indicate there was a biological factor, such as a *Wolbachia*-infected arthropod that cohabitated with *An. gambiae*, in this region and time producing amplicon positive signals. As low prevalence, low intensity infections are challenging to accurately detect it is not possible to conclude with certainty that the *Wolbachia* detected in this study, or our earlier published studies (Baldini et al., 2014; Shaw et al., 2016) are native infections of *Anopheles*.

Author's Contributions

SPS, GLH, and RKD designed the investigations; SPS coordinated the field collection; SPS DAK and AH performed laboratory analysis of samples, SPS, SQ, EBT and OG undertake the phylogenetic Analysis; SPS, GLH, HR and RKD wrote the manuscript with the inputs from DAK, EBT, AH, SQ, OG and AD. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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289

290 **Table**

291 Table1: Spatial and temporal variation of Wolbachia infection prevalence in Burkina
292 Faso

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294 Supplementary Table 1: Details of mosquito samples from Burkina Faso tested for
295 the presence of Wolbachia at LSTM

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297 **Figures**

298 Figure 1: Map of Burkina Faso showing mosquito sampling sites.

299

300 Figure 2: Phylogenetic Analysis of *Wolbachia* Strain. The green colour represents
301 the sequences found in our samples, whilst red colour represents sequences
302 previously identified in *Anopheles gambiae*.

303

304 Supplementary Figure 1: Single-nucleotide variant alignment of all sequences from
305 *Anopheles gambiae*, and *Wolbachia* of *Aedes albopictus*.

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Burkina Faso in Africa

0 100 km

A

B

Table 1: Spatial and temporal variation of *Wolbachia* infection prevalence in Burkina Faso

Year	Village	Species	Tested	Amplicons negative	Amplicons positive (First nested PCR)	Amplicons positive (repeated Nested PCR)
2006	Soumouosso	<i>An. gambiae</i>	331	310	24 (7.25%)	21 (6.34%)
	VK7	<i>An. coluzzii</i>	176	170	19 (11.18%)	6 (3.41%)
2011	VK3	<i>An. coluzzii</i>	506	506	0	0
2015	Soumouosso	<i>An. gambiae</i>	20	20	0	0
2016	Soumouosso	<i>An. gambiae</i>	562	562	0	0
	VK5	<i>An. coluzzii</i>	868	867	1 (0.16%)	0
2018	Kongoussi	<i>An. coluzzii</i>	3	3	0	0
	Soumouosso	<i>An. gambiae</i>	237	237	0	0
	VK3	<i>An. coluzzii</i>	132	130	2 (1.52%)	2 (1.52%)
	VK5	<i>An. coluzzii</i>	853	853	0	0
	VK7	<i>An. coluzzii</i>	1200	1200	0	0
	Yilou	<i>An. coluzzii</i>	65	65	0	0
Total			4953	4923	46 (0.93%)	29 (0.59%)