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2 A Microsporidian blocks *Plasmodium falciparum* transmission in *Anopheles arabiensis* 3 mosquitoes

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22 **Malaria imposes an enormous burden on sub-Saharan Africa, and evidence that incidence**
23 **could be starting to increase again[1] suggests the limits of currently applied control strategies**
24 **have now been reached. A possible novel control approach involves the dissemination in**
25 **mosquitoes of inherited symbiotic microbes to block transmission. This strategy is**
26 **exemplified by the use of transmission-blocking *Wolbachia* in *Aedes aegypti* against dengue**
27 **virus[2-7]. However, in the *Anopheles gambiae* complex, the primary African vectors of**
28 **malaria, there limited reports of inherited symbionts with transmission-blocking capacity[8-**
29 **10]. Here we show that a newly discovered vertically transmitted species of *Microsporidia***
30 **symbiont in the *An. gambiae* complex blocks *Plasmodium* transmission. *Microsporidia MB* is**
31 **present at moderate prevalence in geographically dispersed populations of *An. arabienesis***
32 **in Kenya, localized to the mosquito midgut and ovaries, and is not associated with significant**
33 **reductions in adult host fecundity or survival. Field collected *Microsporidia MB*-infected *An.***
34 ***arabiensis* were never found to harbor *P. falciparum* gametocytes and on experimental**
35 **infection with *P. falciparum* no sporozoites could be detected in *Microsporidia MB*-infected**
36 **mosquitos. As a *Plasmodium* transmission-blocking microbe that is non-virulent and**
37 **vertically transmitted, *Microsporidia MB* could be exploited as a novel malaria control tool.**

38 Microsporidia are a group of obligately intracellular simple eukaryotes, classified within or as a
39 sister group to fungi, and found in a wide range of hosts, but most commonly invertebrates. Their
40 lifecycles include a meront phase during proliferation, and spores with chitinous cell walls
41 involved in host to host transmission through spore ingestion. Species with solely horizontal
42 transmission usually show greater virulence and lower host specificity, but where a combination
43 of horizontal and vertical transovarial transmission occurs, lower virulence is advantageous, and
44 is normally associated with a higher degree of host specificity[11-12]. Sex ratio distortion toward

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45 females has been reported (a manipulation characteristic of transovarially transmitted symbionts),
46 for example *Dictyocoela* microsporidia in Amphipod crustaceans[13]. Various microsporidia species
47 have been reported in mosquitoes[14-25], with simple or complex lifecycles[14] but all of which are
48 pathogens where virulence is primarily associated with larval mortality or reduced adult fecundity
49 and lifespan[14-20].

50 In this study a previously unknown species of *Microsporidia* was discovered during microbiome
51 characterization of *An. arabiensis,* designated *Microsporidia MB*, and was found to occur at a high
52 density and low to moderate prevalence (0-10%) in geographically dispersed populations of *An.*
53 *arabiensis* in Kenya. Notably, in all *An. arabiensis* populations investigated, we observed that
54 none of the *Microsporidia MB* harboring mosquitoes were infected with *Plasmodium* (Extended
55 Data Fig. 1). Phylogenetic analysis of the 18S ribosomal gene revealed that *Microsporidia MB* is
56 related to *Crispospora chironomi*[26], a species recently identified from non-biting midges. The 18S
57 gene sequence of *Microsporidia MB* shows 97% similarity with *Crispospora chironomi*.
58 *Microsporidia MB* and *Crispospora chironomi* are in clade IV that unites microsporidia of
59 terrestrial origin infecting diverse hosts[27] (Fig. 1A and Extended data Fig. 1). The previous reports
60 of microsporidia infecting *Anopheles* mosquitoes all belong to different clades of *Microsporidia*.
61 The morphology of *Microsporidia MB* closely resembles *Crispospora chironomi,* exhibiting both
62 polysporoblastic and diplosporoblastic sporogenies, both found in the larval mosquito gut

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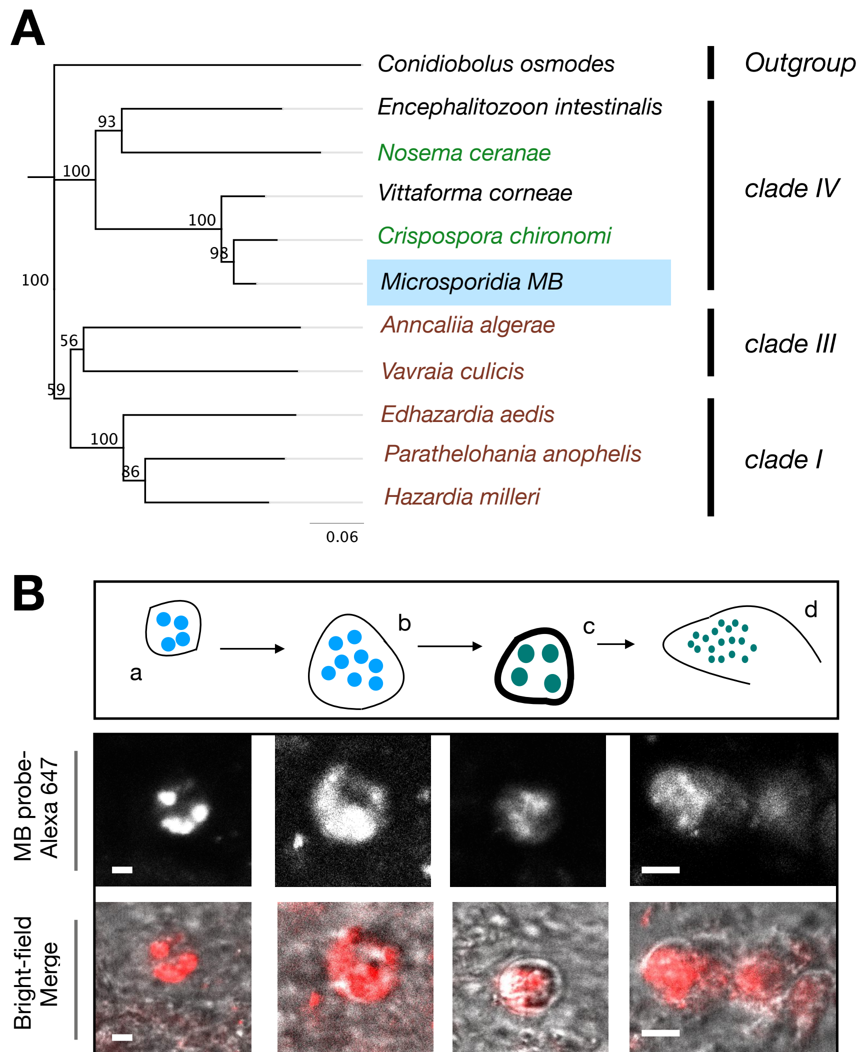
63 epithelium (Fig. 1B).



64

65 **Figure 1: Novel Microsporidian associated with *An. arabiensis* populations in Kenya.** (A) 18S rDNA-based phylogeny reveals
66 that *Microsporidia MB* are in clade IV of the Microsporidia. Labelled in brown are the microsporidian species known from
67 mosquitoes. In green are microsporidians associated with other insect groups. (B) FISH staining of the diplokaryotic stages of
68 *Microsporidia MB* merogony (a-b) and spore capsule formation (c-d) in *An. arabiensis* larval gut epithelial tissues. Scale bar,1.5
69 µm.

70 The effects of *Microsporidia MB* on *Plasmodium* infection in *An. arabiensis* was further examined

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71 around Mbita point, western Kenya, using G_1 offspring pools obtained from field-collected G_0
72 mosquitoes and membrane feeding assays (MFAs) carried out with *Plasmodium falciparum*
73 infected donor blood. Since there was a low to moderate prevalence of *Microsporidia MB* is field
74 populations (Extended Data Fig. 2), we screened mosquitoes for *Microsporidia MB* and sorted
75 them to ensure that G_1 progeny pools used for MFAs would have both *Microsporidia MB*-infected
76 and uninfected mosquitoes. *Plasmodium* was quantified 10 days after MFAs, and a strong negative
77 correlation was apparent between the *Microsporidia MB* and the presence of *Plasmodium* in whole
78 mosquitoes 10 days after MFA (Fig. 2A-B). *Plasmodium* parasites in an ingested bloodmeal
79 undergo a series of developmental changes prior to traversing the peritrophic matrix and midgut
80 epithelium to form a sporogenic oocyst, which releases sporozoites into the hemocoel. From the
81 hemocoel, sporozoites travel to the salivary gland, traverse an epithelium and mix with *Anopheles*
82 saliva resulting in an infectious mosquito, usually 8-14 days after the blood meal[28]. To investigate
83 the stage at which *Plasmodium* development was inhibited, we specifically quantified *Plasmodium*
84 in the *Anopheles arabiensis* head and thorax and abdominal compartments. The absence of
85 *Plasmodium* in the head and thorax compartment of *Microsporidia MB* infected *Anopheles*
86 *arabiensis* 10 days after MFA indicates that *Microsporidia MB* prevents *Anopheles arabiensis*
87 salivary glands from being colonized by *Plasmodium* sporozoites, and therefore prevents
88 *Plasmodium* transmission (Fig. 2C-D). In addition, no *Plasmodium* oocysts are established in the
89 midgut of *Microsporidia MB* infected *Anopheles arabiensis* as determined by the absence of
90 *Plasmodium* infections in abdomens 10 days after MFA (Extended data Fig. 3). These results
91 indicate that *Microsporidia MB*-induced transmission blocking occurs prior to the establishment

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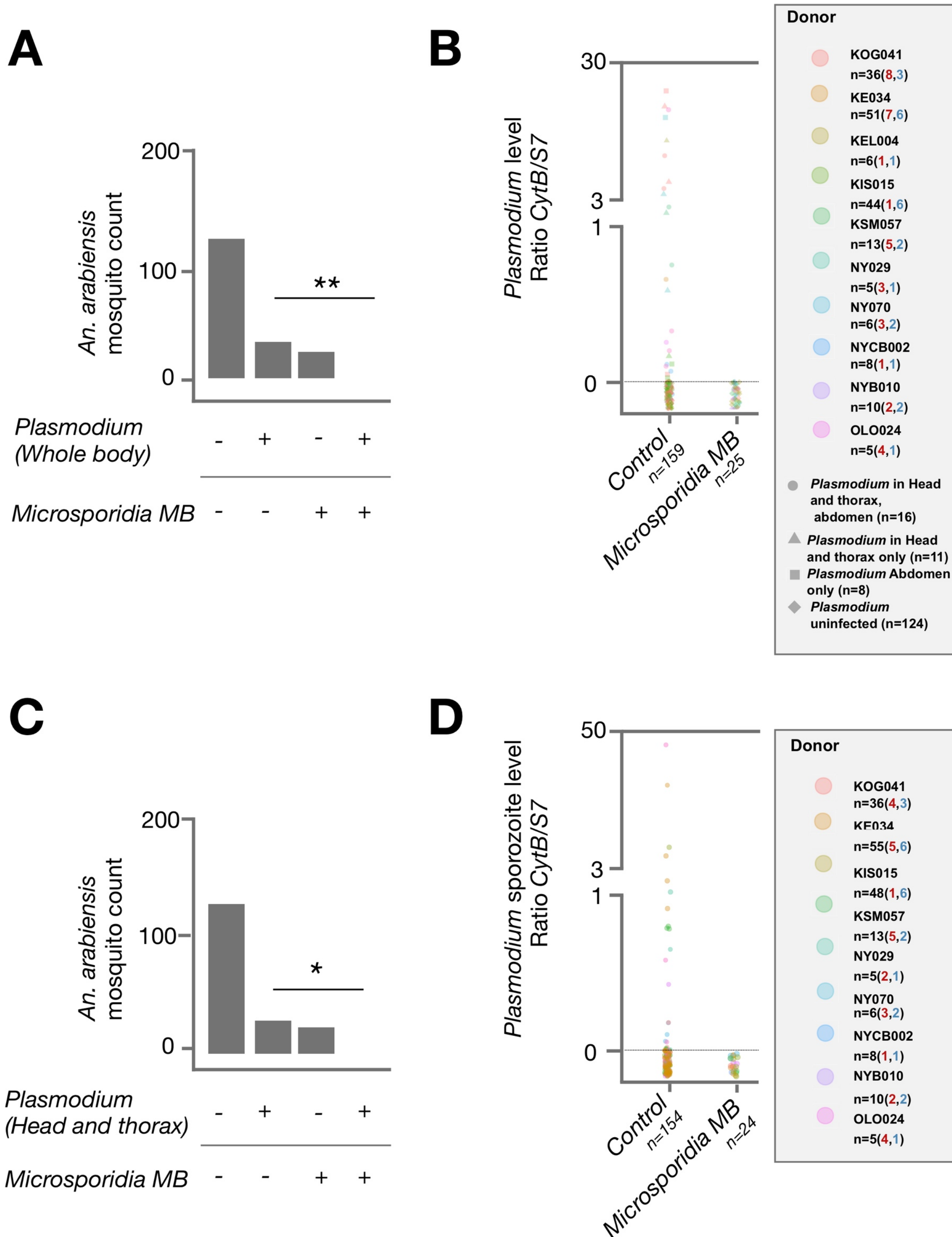
92 of oocysts in the *Anopheles* mosquito midgut.



93

94 **Figure 2: *Microsporidia MB* blocks parasite development in *An. arabiensis* after membrane feeding assay challenge with *P.***

95 ***falciparum***. (A) The *Plasmodium* infection rate in *Microsporidia MB* positive and *Microsporidia MB* negative mosquitoes was

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96 determined by qPCR. There was a significant absence of co-infected mosquitoes (two tailed fisher exact test, P=0.005 N=184). (B)
97 *Plasmodium* density quantified by qPCR in *An. arabiensis*. For *An. arabiensis* mosquitos infected in both head and thorax and
98 abdomen, average *Plasmodium* density across both compartments is given. (C) The head and thorax *Plasmodium* infection rate,
99 reflecting presence of sporozoites, in *Microsporidia MB* positive and *Microsporidia MB* negative mosquitoes. There was a
100 significant absence of co-infected mosquitoes (two tailed fisher exact test, P=0.02 N=178). (D) *Plasmodium* density in *An.*
101 *arabiensis* heads and thoraxes, quantified by qPCR in. Data shown in A,B, C, D is pooled from replicate experiments carried out
102 using different gametocyte donors (for each donor the numbers of *Plasmodium* positive *An. arabiensis* is shown in red and
103 *Microsporidia MB* positives *An. arabiensis* is shown in blue). Each data point is an individual *An. arabiensis* mosquito. If
104 *Plasmodium* was detected in either head and thorax or abdomens or both compartments, the *An. arabiensis* was considered to have
105 a *Plasmodium* infection (A,B). Heads and thoraxes were separated and screened individually for head and thorax specific infection
106 rate (C,D).

107 *Microsporidia MB* are generally maternally transmitted with high efficiency (Fig. 3A), from 45-
108 100%. However, a number of wild-caught females with low *Microsporidia MB* density did not
109 transmit or transmitted very poorly to their offspring. It is possible that these infections might be
110 newly acquired and have not yet become localized to the ovaries, a likely requirement for high-
111 efficiency maternal transmission. *Microsporidia MB* were observed in the mosquito ovaries, where
112 the symbiont colonizes and penetrates oocytes (Fig. 3B).

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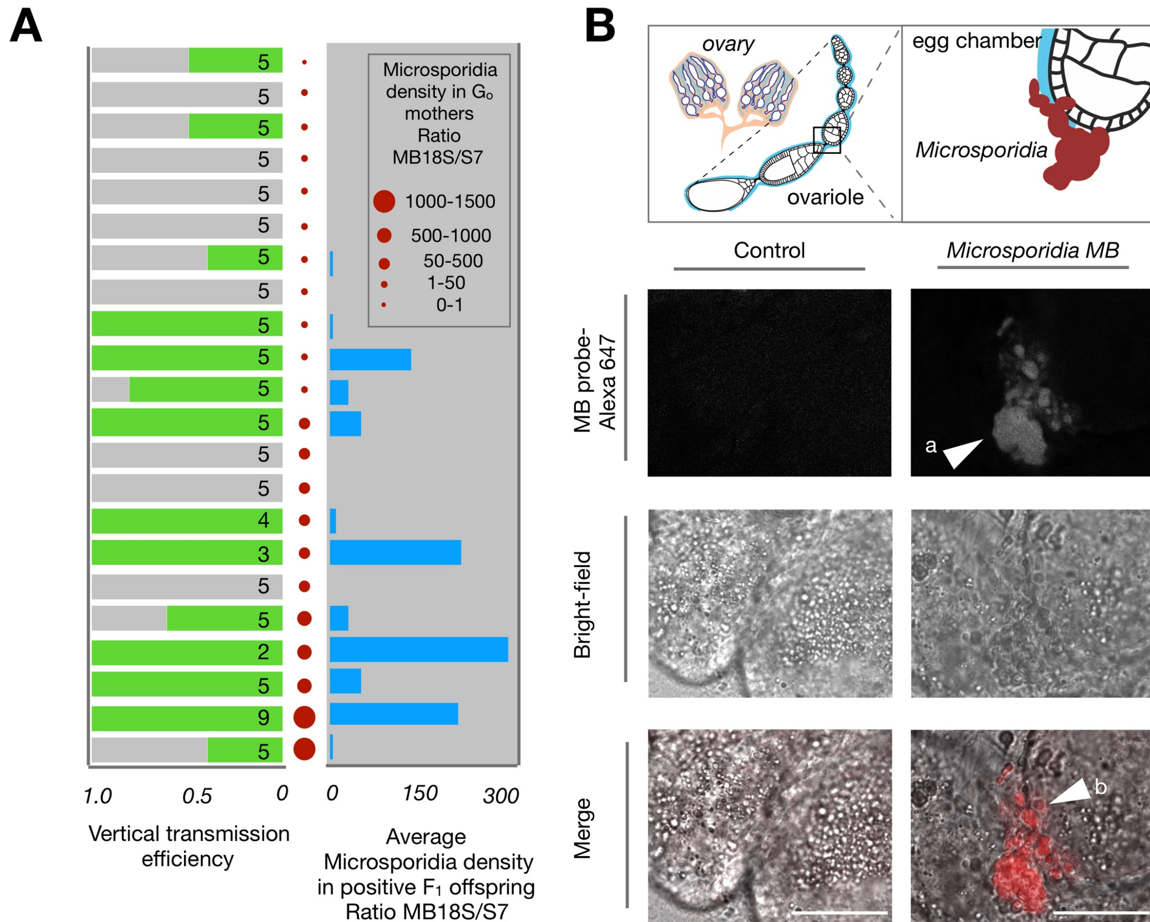

113

114 **Figure 3: *Microsporidia MB* is maternally transmitted**. (A) The vertical transmission efficiency and density of *Microsporidia*
115 *MB* in G_1s depends on the G_0 (maternal) *Microsporidia MB* density. Each red dot represents a *Microsporidia MB*-infected G_0
116 female *Anopheles arabiensis* mosquito. The density of *Microsporidia MB* in G_0 females is reflected by the size of the red dot.
117 Green bars depict the vertical transmission efficiency from G_0 to G_1, whereas blue bars depict the density of *Microsporidia MB* in
118 G_1s. Numbers of G_1s tested to determine vertical transmission efficiency and *Microsporidia MB* density are given in the bars. (B)
119 Fluorescence microscopy with a *Microsporidia MB*-specific FISH probe (MB probe) indicates that *Microsporidia MB* (a) is
120 localized to the posterior of developing vitellogenic egg chambers (b) in *An. arabiensis*. Scale bar, 20 µm.

121 The fecundity and egg to adult survival rate of *Microsporidia MB* infected and uninfected iso-
122 female lineages were then examined. No significant differences were observed in the number of
123 eggs laid by *Microsporidia MB* infected versus uninfected individuals, indicating
124 that *Microsporidia MB* does not have a sterilizing effect on females (Fig. 4A). A shortened

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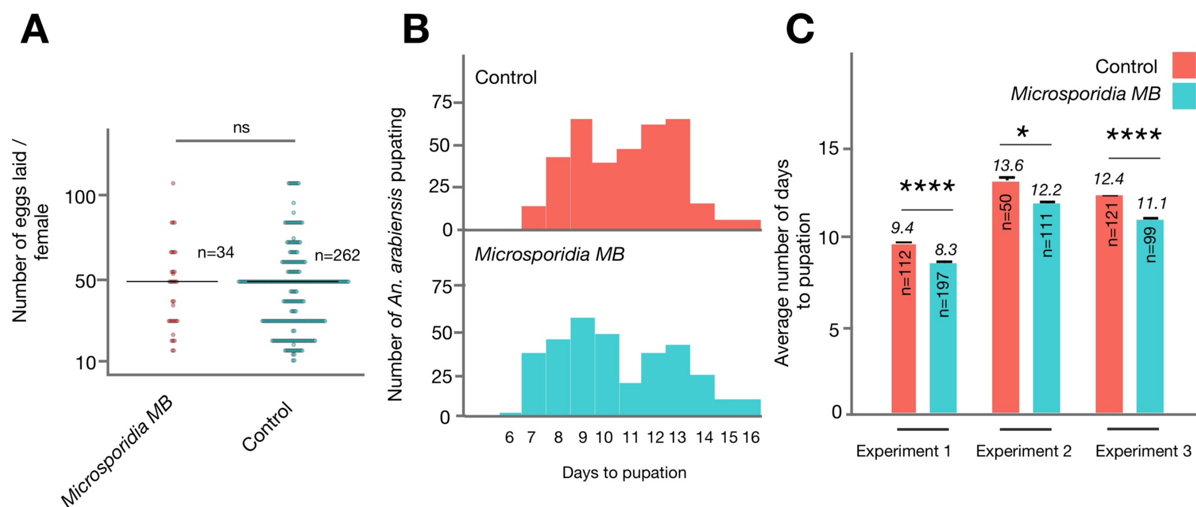
125 development period from egg to adult was observed for individuals carrying *Microsporidia MB*
126 (Fig. 4B). We investigated the survival of adult female mosquitoes harboring *Microsporidia MB*
127 and found their longevity was similar to uninfected mosquitoes (Extended Data Fig. 3). The
128 density of *Microsporidia MB* was examined across the lifecycle of the mosquito and was higher
129 in adults than in larvae, but larval density is highly variable. Notably, *Microsporidia MB* levels are
130 lower in recently emerged adults, increasing with mosquito age (Extended Data Fig. 3).



131

132 **Figure 4: *Microsporidia MB* does not overtly decrease host fitness**. (A) *Microsporidia MB*-harboring wild-caught mosquitoes
133 did not lay significantly less eggs than uninfected counterparts. Black line indicates means. (B) The F_1 larval progeny of
134 *Microsporidia MB*-infected wild-caught females develop significantly faster than uninfected counterparts, data shown is pooled
135 from three independent experiments. (C) The larval development of *Microsporidia MB*-infected *An. arabiensis* is on average 1.1,
136 1.4 and 1.3 days less than uninfected controls in three independent experiments, error bars reflect SEM.

137 Several *Anopheles*-associated microsporidians have been shown to interfere with the infection and
138 development of *Plasmodium*. *Nosema stegomyiae* disrupts the development of the oocysts in *An.*
139 *gambiae*, attributed to mid-gut degradation and consequent disruption of *Plasmodium* binding[24],
140 while *Vavraia culicis* inhibition of development of *Plasmodium* has been associated with host
141 innate immune priming[25]. The inhibitory effects observed in previous *Plasmodium* transmission

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142 experiments have been relatively modest, with only partial reduction of transmission observed. In
143 applied terms, given their virulence, their primary application would be as population suppression
144 agents and the *Plasmodium* inhibition would provide a minor add-on. In contrast, complete *P.*
145 *falciparum* transmission blocking was observed here for *Microsporidia MB*. Additionally,
146 *Microsporidia MB* blocking occurs early, prior to the formation of *Plasmodium* oocysts in the
147 *Anopheline* mosquito gut. When combined with the key characteristics that will facilitate
148 artificially elevating its population frequency, namely spore production that is likely to facilitate
149 dissemination, efficient transovarial transmission and apparently non-virulent interactions with
150 *An. arabiensis* mosquitoes, *Microsporidia MB* is a realistic candidate for a stable vector population
151 replacement strategy for malaria control.

152 These findings are significant in terms of regional malaria transmission and epidemiology as well
153 as risk-mapping, and particularly in terms of the development of microbe-
154 based *Plasmodium* transmission blocking tools. *Microsporidia MB* can be added to
155 the *Anopheles*-associated gut bacteria that have previously been identified as able to
156 reduce *Plasmodium* transmission when introduced into the mosquito[29,30], or following
157 introduction of anti-*Plasmodium* transgenes[31]. As an unmodified, *Anopheles*-associated inherited
158 endosymbiont that confers highly effective malaria blocking under natural conditions, it is
159 a promising prospect for malaria control.

160

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230

231 **Materials and Methods**

232

233 **Ethics statement**

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234 Ethical clearance (Kenya Medical Research Institute Scientific and Ethics Review Unit:
235 KEMRI/RES/7/3/1 and Glasgow MLVS College Ethics Committee: Project Number 200170001)
236 was obtained prior to human blood sample collection. Written informed consent was sought from
237 parents and guardians of the children to allow minors to participate in the study. Consent was also
238 obtained from heads of households that provided approval for indoor mosquito collection.

239

240 **Data and materials availability statement**

241 All data is available in the main text or the extended data.

242

243 **Sampling sites and collection**

244 *Microsporidia MB* and *Plasmodium* prevalence in wild *Anopheles arabiensis* mosquitoes was
245 determined by collecting adult female mosquitoes from sites around Kenya: Mbita (Nyawiya,
246 Mageta and Kirindo), Mwea (Mbui-Njeru), Busia (Funyala) and Ahero (Kigoche). *An. arabiensis*
247 were collected inside houses and sheds using CDC light traps and by manual aspiration. For the
248 establishment of *Microsporidia MB* harboring lines gravid mosquitoes were collected solely by
249 manual aspiration inside houses and sheds. All mosquitoes were transported from the field to the
250 *icipe*-TOC laboratories and insectaries alive in cages, each sample represents an individual wild
251 caught mosquito.

252

253 **Mosquito species identification**

254 All experiments were carried out on wild collected *Anopheles gambiae sl.,* which were identified
255 morphologically. In all of the collection sites, *Anopheles arabiensis* is the most common member
256 of the *An. gambiae* species complex, with >97% of complex members being identified as

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257 *Anopheles arabiensis*. The species designation was confirmed using a molecular assay that
258 differentiates *An. gambiae s.s.* and *An. arabiensis* using the SINE S200 X6.1 locus[31]. *Anopheles*
259 samples that were not confirmed to be *Anopheles arabiensis* were excluded from analysis.
260

261 **Determination of Molecular Phylogeny of *Microsporidia MB***

262 *Microsporidia MB* positive *An. arabiensis* were initially identified by sequencing 18S amplicons
263 amplified by the SSU rRNA primer pair F: 5′-CACCAGGTTGATTCTGCC-3′; R: 5′-
264 TTATGATCCTGCTAATGGTTC-3′, which targets phylogenetically diverse microsporidians[32].
265 The primer pair RPOBMBF 5′-ACAGTAGGTCACTTGATTGAATGTC-3′ and RPOBMBR 5′-
266 TACCATGTGCTTAAGTCTTTGGT-3′ was used to amplify the *rpoB* gene of *Microsporidia MB*.
267 Amplicaons were prepared from nine individual *An. arabiensis* from geographically dispersed
268 sites, all individuals had identical 18S and *rpoB* fragment gene sequences. Prepared amplicons
269 were cleaned using the USB® ExoSAP-IT® PCR Product Cleanup kit according to manufacturer's
270 instructions and sent to Macrogen (Netherlands) for sequencing. Multiple sequence alignment was
271 done using the MUSCLE algorithm[33] alongside reference sequences of other *Microsporidia*
272 species obtained from NCBI. Tamura-Nei genetic distance model alongside Neighbour-joining
273 tree building algorithm was used in the creation of phylogenies and evaluated with 10000
274 replicates bootstrap support and 50% support threshold. Rooting was done using *Conidiobolus*
275 *osmodes* as an outgroup. *Microsporidia MB* 18S rDNA and *rpoB* partial gene reference sequences
276 have been submitted to Genbank, submission ID 2270798.
277

278 **Egg laying and establishment of lines**

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279 Wild-caught gravid female mosquitoes were induced to oviposit inside a perforated 1.5ml micro
280 centrifuge tube containing 50µl of distilled water and a soaked piece of Whatman paper towel.
281 Eggs from each female were counted under a compound microscope using a paint brush and then
282 dispensed into water tubs for larval development under optimal rearing conditions (a temperature
283 of 30.5 °C and 30% humidity). Upon laying eggs, the G_0 females were screened for presence of
284 *Microsporidia MB* by PCR. The number of eggs laid by *Microsporidia MB* infected and uninfected
285 mosquitoes was recorded and two-tailed Mann Whitney U tests were used to determine statistical
286 significance. The progeny from *Microsporidia MB* positive G_0 females were used for: i) the
287 quantification of vertical transmission efficiency and ii) the establishment of iso-female
288 *Microsporidia MB* infected lines, whereas lines with similar L1 larvae numbers (+/- 10
289 individuals) were used as matched negative the controls for quantifying development time and
290 survival. For each iso-female line two G_1 L4 larvae were screened to determine species and
291 confirm infection status and the line was only considered infected if both were *Microsporidia MB*
292 positive. For the quantification of density across developmental stages five infected iso-female
293 lines were established, two of which were monitored until day eight and three of which were
294 monitored until day fifteen. All the mosquitoes in each experiment were derived from a single
295 infected female. To establish mixed (*Microsporidia MB* positive and negative) *An. arabiensis*
296 pools for standard membrane feeding assays, eggs from *Microsporidia MB*-infected and
297 uninfected females were combined and reared together at an approximate ratio of 3:1
298 (*Microsporidia MB infected*: *Microsporidia MB uninfected*). Infection status was not determined
299 at the larval stage for mixed pools.

300

301 **Mosquito rearing**

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302 Larvae were reared under a controlled environment at 30.5 °C (+/- 2°C). Larvae were fed daily on
303 TetraMin™ baby fish food and fresh double-distilled water added into their tubs every other day
304 to maintain oxygen levels. Adult mosquitoes were reared at 30°C (+/- 2°C) and 70% humidity with
305 a constant 12-hour day / night cycle. The adult mosquitoes were fed on 6% glucose soaked in
306 cotton wool. For adult survival and larval development time measurements, the status of larvae
307 and mosquitoes was recorded every 24hrs, the Log-Rank and two-tailed Mann Whitney U tests
308 were used to determine levels of statistical significance, respectively.
309

310 ***Plasmodium* standard membrane feeding assays**

311 *Plasmodium* screening of human subjects was done in the regions surrounding Mbita using RDT
312 kits (SD Bioline, UK). Microscopy was carried out on RDT-positive samples to confirm the
313 presence of *P. falciparum* gametocytes. Gametocyte-positive blood used was mixed with an
314 anticoagulant (heparin) and a total volume of 100µl was placed into mosquito mini-feeders at 37°C
315 and covered in stretched parafilm. Mixed pools of 2-3 day old *An. arabiensis* (containing co-reared
316 *Microsporidia MB* positive and negative mosquitoes) were starved for 5 hours (the sucrose source
317 was replaced with water for the first 4 hours of starvation) prior to the standard membrane feeding
318 assay. Mosquitoes were allowed to feed for an hour after which non blood-fed individuals were
319 discarded. Blood-fed mosquitoes were then maintained for a period of 10 days post-infection and
320 processed for the molecular detection of *Microsporidia MB* and *Plasmodium* oocysts and
321 sporozoites. We only include experiments where there was greater than zero prevalence of
322 *Microsporidia MB* and *Plasmodium* in the mixed pools of *Anopheles arabiensis.* Two-tailed fisher
323 exact tests were used to determine statistical significance. Each data point represents an individual
324 mosquito.

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325

326 **Specimen storage and DNA extraction**

327 All *An. arabiensis* specimens were dry frozen at -20°C in individual microcentrifuge tubes prior
328 to DNA extraction. Prior to extraction, mosquitoes were sectioned into head and thorax (for
329 detection and quantification of *Plasmodium* sporozoites) and abdomens (for detection and
330 quantification of *Plasmodium* oocysts and *Microsporidia MB*). DNA was extracted from each
331 section individually using the protein precipitation method (Puregene, Qiagen, Netherlands).

332

333 **Molecular detection and quantification of *Microsporidia MB***

334 *Microsporidia MB*-specific primers (MB18SF: CGCCGGCCGTGAAAAATTTA and MB18SR:
335 CCTTGGACGTGGGAGCTATC) were designed to target the *Microsporidia MB* 18S rRNA gene
336 region and tested for specificity on a variety of Microsporidia-infected mosquito controls
337 (including *Hazardia, Parathelohania* and *Takaokaspora*). For detection, the PCR reaction volume
338 was 10µl, consisting of 2µl HOTFirepol® Blend Master mix Ready-To-Load (Solis Biodyne,
339 Estonia, mix composition: 7.5 mM Magnesium chloride, 2mM of each dNTPs, HOT FIREPol®
340 DNA polymerase), 0.5µl of 5 pmol/µl of both forward and reverse primers, 2 µl of the template
341 and 5 µl nuclease-free PCR water. The PCR cyclic conditions used were; initial denaturation at
342 95°C for 15 minutes, further denaturation at 95°C for 1 minute, followed by annealing at 62°C for
343 90 seconds and extension at 72°C for a further 60 seconds, all done for 35 cycles. Final elongation
344 was done at 72°C for 5 minutes. *Microsporidia MB* was also quantified by qPCR using MB18SF/
345 MB18SR primers, with normalization against the *Anopheles* ribosomal S7 host gene (primers,
346 S7UF: GMCGGGTCTGWACCTTCTGG and S7UR: TCCTGGAGCTGGAARTGAAC). The
347 qPCR reaction volume was 10µl, consisting of 2 µl HOT FIREPol® EvaGreen® HRM no ROX

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348 Mix (Solis Biodyne, Estonia, mix composition: 12.5 mM Magnesium chloride, EvaGreen® dye,
349 BSA, dNTPs, HOT FIREPol® DNA Polymerase and 5× EvaGreen® HRM buffer), 0.5µl of 5
350 pmol/µl of both forward and reverse primers, 2 µl of the template and 5 µl nuclease-free PCR
351 water. Finally, a melt curves were generated including temperature ranges from 65°C to 95°C.
352 Standard curves were also generated to determine amplification efficiency.
353

354 **Molecular detection and quantification of *Plasmodium***

355 A qPCR-based assay was used to detect and quantify the *cytochrome b* gene of *Plasmodium*[34].
356 When utilized on *An. arabiensis* head and thorax DNA samples 10 days post MFA this assay can
357 be used to detect and quantify *Plasmodium* sporozoites. When utilized on *An. arabiensis* abdomen
358 DNA samples 10 days post MFA this assay can be used to detect and quantify *Plasmodium*
359 oocysts. Plasmodium *cytochrome b* was normalized against the *Anopheles* ribosomal S7 host gene.
360 The qPCR mastermix was composed of 2 µl HOT FIREPol® EvaGreen® HRM no ROX Mix (Solis
361 Biodyne, Estonia, mix composition: 12.5 mM Magnesium chloride, EvaGreen® dye, BSA, dNTPs,
362 HOT FIREPol® DNA Polymerase and 5× EvaGreen® HRM buffer), 0.5 µl of 5 pmol/µl of both
363 forward (cytBF) and reverse (cytBR) primers, 2 µl of the template and 5 µl nuclease-free PCR
364 water. The PCR profile for target gene amplification included an initial denaturation at 95 °C for
365 15 minutes, further denaturation at 95 °C for 30 seconds, followed by annealing at 60 °C for 45
366 seconds and extension at 72 °C for 45 seconds repeated for 40 cycles. Final elongation was
367 performed at 72 °C for 7 minutes followed by generation of a melt curve ranging from 65 °C to 95
368 °C. A standard curve was generated to determine the PCR efficiency.
369

370 **Fluorescence *in situ* Hybridisation (FISH)**

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371 DNA probes specific to *Microsporidia MB* 18S rDNA were designed and synthesized (5'-CY5-
372 CCCTGTCCACTATACCTAATGAACAT-3', Macrogen, Netherlands). FISH was conducted on
373 *An. arabiensis* adult and larval tissue specimens using a previously described protocol[35], with
374 minor modifications. Briefly, mosquito tissues (larval gut and adult ovaries) were fixed in 4%
375 Paraformaldehyde (PFA) solution overnight at 4°C and subsequently transferred into 10%
376 hydrogen peroxide in 6% alcohol for 3 days prior to rehydration in Phosphate Buffer Saline with
377 Tween-20 (PBS-T) for 1–2 hours. Hybridization was conducted by incubating tissues in 150 µl
378 hybridization buffer (20 mM Tris-HCl [pH 8.0], 0.9M NaCl, 0.01% sodium dodecyl sulfate, 30%
379 formamide) containing 100 pmol/ml of the probe at room temperature overnight. After washing
380 with PBS-T, the hybridized samples were placed on a slide and were visualized immediately using
381 a Leica SP5 confocal microscope (Leica Microsystems, USA).

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387 throughput. *Nucleic Acids Res*. **32**(5):1792-7 (2004).
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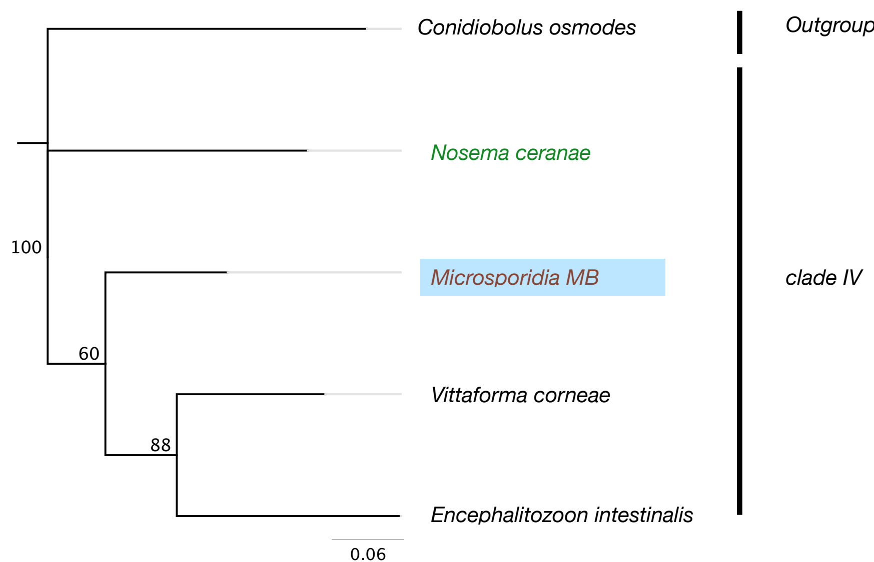
392 detection of endosymbionts. *Applied Entomology and Zoology*. **44**(2):281–91 (2009).
393

394 **Extended Data**



395

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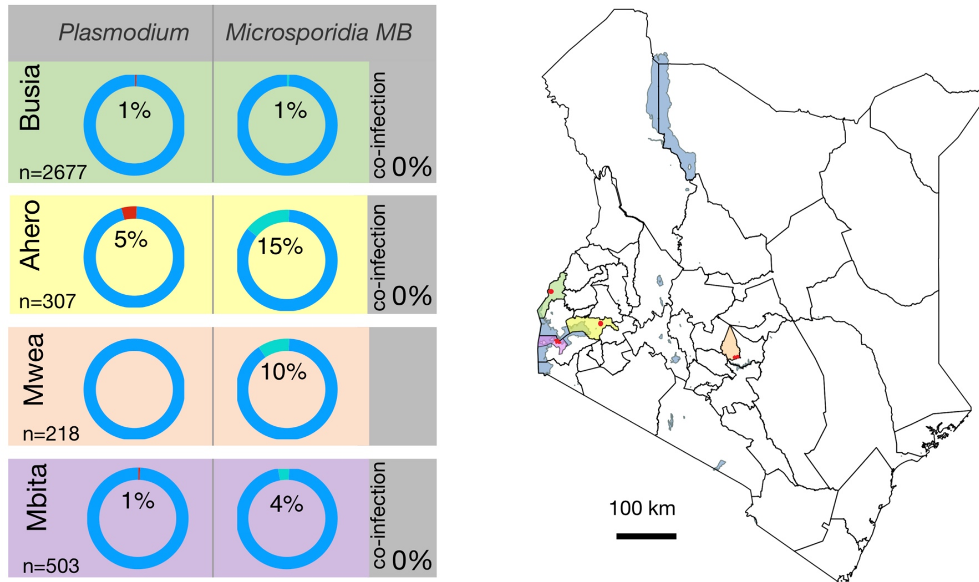
396 **Extended Data Figure 1: The RNA polymerase B' subunit gene (*rpoB*) based phylogeny**

397 **reveals that Microsporidia MB are in clade IV of the Microsporidia.**



398

399 **Extended Data Figure 2: *Microsporidia MB* is found in geographically dispersed *An.***

400 ***arabiensis* populations.** *Microsporidia MB* is found a prevalence range of 1-15% in *An. arabiensis*

401 populations that also have a *Plasmodium* sporozoite infection prevalence range of 1-5%.

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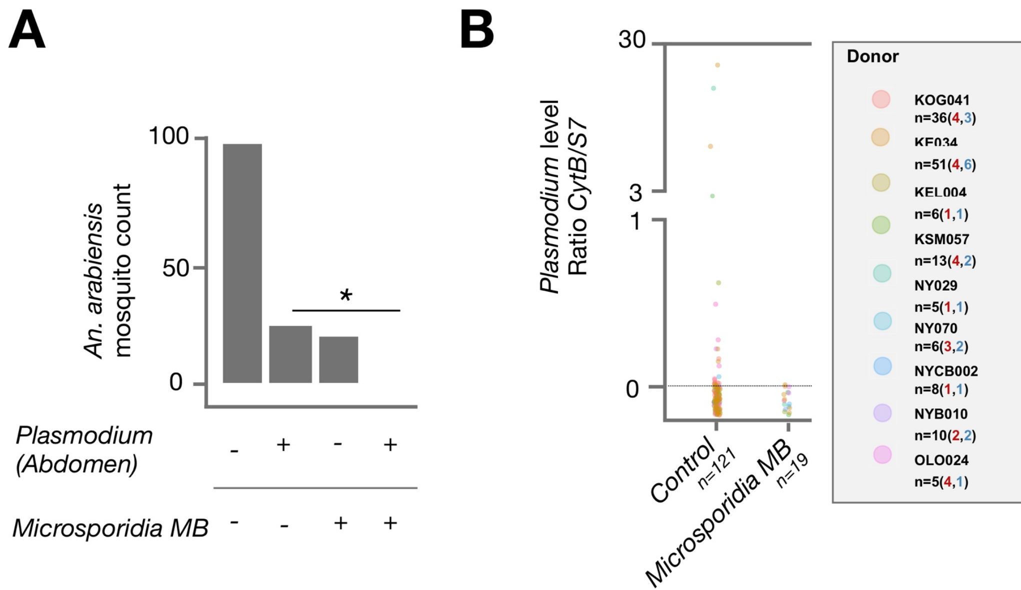
402 *Microsporidia MB* and *Plasmodium* sporozoite co-infections were not observed. The color-coded

403 locations of sampling sites are indicated on the map of Kenya.



404

405 **Extended Data Figure 3: *Microsporidia MB* blocks oocyst formation in *An. arabiensis* after**

406 **membrane feeding assay challenge with *P. falciparum*. The abdomen *Plasmodium* infection**

407 **rate, reflecting presence of oocysts, in *Microsporidia MB* positive and *Microsporidia MB***

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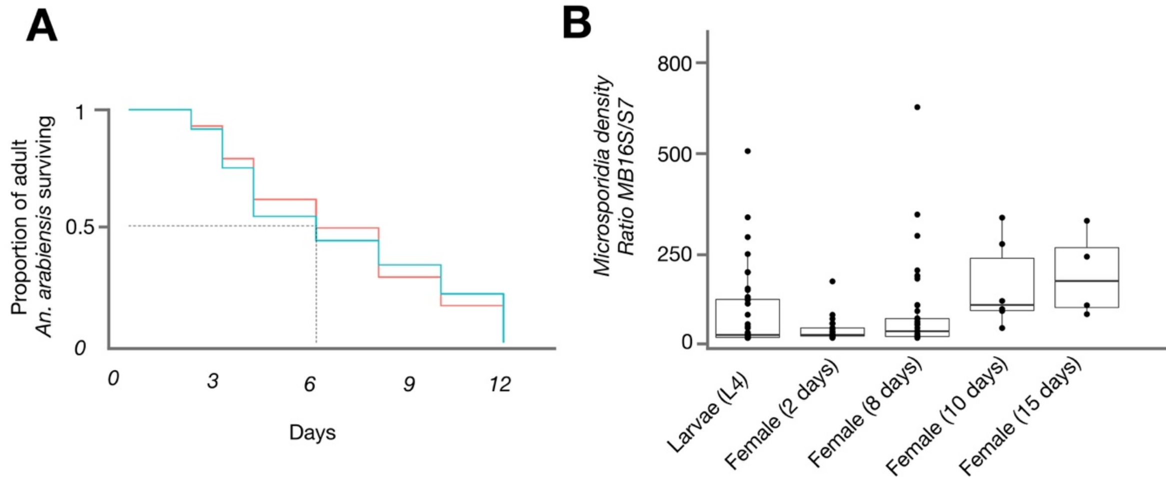
408 **negative mosquitoes. There was a significant absence of co-infected mosquitoes (two tailed**
409 **fisher exact test, P=0.04 N=140).**



410

411 **Extended Data Figure 4:** (A) The survival of adult G_1 progeny of *Microsporidia MB* infected
412 wild-caught females is not significantly different from uninfected counterparts, one experiment
413 (ii) shown of three independent experiments (i-iii); (i, P=0.86, N>9, ii, P=0.97, N>41, ii, P=0.06
414 N>50, N denotes the minimum number of mosquitoes per condition). (B) The density of
415 *Microsporidia MB* across different developmental stages. The density of *Microsporidia MB* is
416 highly variable in larvae, lower in young adults but increase as adult *An. arabiensis* females age.
417 Boxplot boundaries reflect the inter-quartile range. Each datapoint represents an individual
418 mosquito from a total of five (L4-day 8) and three (day 10 and 15) independent experiments.

419

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428 conceived and designed the majority of the experiments. E.M and L.M. performed the majority of
429 experiments. J.O and E.M. collected mosquitoes, screened them and prepared them for
430 experiments. H.B. and M.V.M. carried out transmission-blocking and molecular identification
431 experiments, respectively. J.K.H., S.P.S., E.M., L.M., M.V.M. and V.A.M. analyzed the data.
432 J.K.H and S.P.S. wrote the manuscript. **Competing interests:** Authors declare no competing
433 interests.
434