bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Gametocyte production and transmission fitness of African and Asian *Plasmodium falciparum* isolates with differential susceptibility to artemisinins

Nicholas I. Proellochs[1], Chiara Andolina[1], Jordache Ramjith[1], Rianne Stoter[1], Geert-Jan van Gemert[1], Wouter Graumans[1], Susana Campino[2], Leen N. Vanheer[2], Martin Okitwi[3], Patrick K. Tumwebaze[3], Melissa D. Conrad[4], Taane G. Clark[2], David A. Fidock[5,6], Didier Menard[7], Sachel Mok[6], Teun Bousema[1,2]

[1] Department of Medical Microbiology, Radboud University Medical Centre, Nijmegen, The Netherlands
[2] Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK
[3] Infectious Diseases Research Collaboration, Kampala, Uganda
[4] Department of Medicine, University of California, San Francisco, CA, USA
[5] Department of Microbiology and Immunology Columbia University Irving Medical Center, New York, NY, USA
[6] Center for Malaria Therapeutics and Antimicrobial Resistance, Division of Infectious Diseases, Department of Medicine, Columbia University Irving Medical Center, New York, NY, USA
[7] Institut Pasteur, Université Paris Cité, Malaria Parasite Biology and Vaccines Unit, Paris, France; Malaria Genetics and Resistance Team (MEGATEAM), UR 3073 - Pathogens Host Arthropods Vectors Interactions, Université de Strasbourg, F-67000 Strasbourg, France; CHU Strasbourg, Laboratory of Parasitology and Medical Mycology, Strasbourg, France; Institut Universitaire de France (IUF), F-75231, Paris, France

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Abstract

The emergence of *Plasmodium falciparum* parasites partially resistant to artemisinins (ART-R) poses a significant threat to recent gains in malaria control. ART-R has been associated with PfKelch13 (K13) mutations, which differ in fitness costs. This study investigates the gametocyte production and transmission fitness of African and Asian *P. falciparum* isolates with different K13 genotypes across multiple mosquito species. We tested three ART-sensitive (ART-S) isolates (NF54, NF135, NF180) and three ART-R isolates (ARN1G, 3815, PAT-023) for sexual conversion and transmission to *Anopheles stephensi, An. gambiae* and *An. coluzzii*. ART-R levels were quantified *in vitro* using the Ring-stage Survival Assay (RSA), and the transmission-reducing effects of dihydroartemisinin (DHA) on mature gametocytes were assessed. Results showed that ART-S parasite lines consistently produced gametocytes and transmitted effectively in all three mosquito species. ART-R isolates showed variability: ARN1G maintained high transmission levels, whereas 3815 showed limited transmission potential despite higher sporozoite loads in *An. coluzzii*. The African ART-R isolate PAT-023 demonstrated low gametocyte commitment but was transmitted efficiently in both *An. gambiae* and *An. coluzzii*. DHA exposure reduced mosquito infectivity for all isolates, regardless of K13 genotype. These findings, based on a limited number of field isolates, suggest that ART-R parasites remain transmissible across different *Anopheles* species. However, K13 mutations do not appear to confer a direct transmission advantage. This study highlights the complexity of ART-R dynamics and underscores the need for further research to inform malaria control strategies in regions where ART-R parasites are circulating.

Key words

Malaria, *P. falciparum*, Mosquito Transmission, Artemisinin Resistance, K13 genotype

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Introduction

Despite global efforts to reduce malaria burden, progress has plateaued in the last decade and in some areas malaria is again increasing [1]. The recent emergence in Sub-Saharan Africa of *P. falciparum* parasites with partial resistance to artemisinins (ART-R) adds to the concerns about how sustainable malaria control may be. ART-R is characterised by prolonged parasite clearance times (half-life >5 hours) or persistence of parasitemia on day 3 following artemisinin monotherapy or artemisinin-based combination therapy (ACT) [2-6]. ART-R is predominantly associated with specific mutations in the essential PfKelch13 (K13) protein's β-propeller domain [7-10]. These mutations reduce K13 protein levels, disrupting haemoglobin import into the parasite's digestive vacuole, a critical step for ART activation [8, 11-13]. The impacts of K13 mutations on both resistance and parasite fitness are variable, depending on the mutation and parasite background [13-16], which makes it difficult to predict which mutations are of particular concern.

Clinically relevant mutations in K13 first were observed in isolates collected in 2002 in western Cambodia [17]. While partial resistance subsequently spread across the Greater Mekong Subregion [7, 18], the anticipated expansion to the African continent has not occurred. It was hypothesised that lower drug pressures combined with high parasite diversity in many settings in Sub-Saharan Africa may have allowed wild-type parasites to outcompete less-fit K13 mutant parasites, making *de novo* emergence of ART-R less likely and limiting the spread of the K13 mutants upon introduction. The recent independent emergence of K13 mutations associated with ART-R including in East Africa (Uganda, Rwanda, and Tanzania) and the Horn of Africa (Eritrea, Ethiopia and Sudan) [19-23] raises questions about their fitness, resistance, and transmission potential. Transmission to mosquitoes depends on the formation of viable male and female gametocytes, with considerable variation in gametocyte production between parasite isolates [24, 25]. The transmission potential of parasites with K13 mutations is particularly relevant with changes in vector populations and variation in *P. falciparum* vector competence [26]. The recent invasion of the competent Asian vector *Anopheles stephensi* in urban African settings [27, 28] raises concerns about the spread of ART-R parasites of African and Asian origin in settings previously not endemic for malaria.

Understanding variations in vector competence and determining whether *K13* mutant parasites possess a transmission advantage, both in the presence and absence of artemisinin derivatives, are crucial for predicting the potential spread of ART-R on the African continent. In this study, we investigate the gametocyte production and transmission fitness of ART-S and ART-R field isolates. We compared ART-S isolates [29, 30] with ART-R isolates carrying two common Southeast Asian K13

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

mutations G449A and C580Y [18, 31, 32], as well as a recently isolated ART-R parasite line from Uganda, where the local emergence of ART-R has been documented.

Material and methods

Parasite cultures

All parasite lines – including the ART-S NF54 (Africa), NF135 (Cambodia) and NF180 (Uganda), as well as the ART-R *P. falciparum* field isolates ARN1G (Thailand), 3815 (Cambodia), and PAT-023 (Uganda) – were cultured in an automated culture system using RPMI media supplemented with 10% human serum [33]. Asexual parasite cultures were maintained in a synchronised state through magnet separation or sorbitol lysis. Gametocyte induction was performed using either minimal fatty acid media or 0.5% Albumax; gametocytes were allowed to mature in RPMI media supplemented with 10% human serum [25]. Parasite DNA was extracted with QIAGEN Blood DNA kit, sequenced on the Illumina Novaseq 6000 platform and analysed using the malaria profiler tool [34].

Ring-stage Survival Assay (RSA)

The Ring-stage Survival Assay (RSA) was performed as previously published [16, 35] with minor modifications. To avoid the negative impact of sorbitol synchronisation on conversion rates within the same cycle [36, 37], we opted for a gentler approach using double magnet purification. Highly synchronised parasites were first passed through a magnetic column (MACS) to collect segmented schizonts. These schizonts were put back into culture for 3-4 hours to allow bursting and reinvasion of new red blood cells (RBCs). The culture was subsequently passed through the same magnetic column, isolating the flow-through, which contained only newly invaded ring-stage parasites. Parasites were split into 2 plates: one for the sexual conversion assay and one for the RSA. For the RSA, parasites were diluted to 1-2% parasitemia in 2% hematocrit. Each line was tested in 4 wells: two for DMSO controls and two for DHA. Washes were performed in separate tubes following DHA treatment before being added to a fresh plate. Survival rates were determined using both Giemsa smears and flow cytometry using MitoTracker Red as a live cell stain.

Sexual conversion assay

Sexual conversion rates were performed as previously published [25], with minor modifications. After double synchronisation, parasites were returned to culture for 24 hours prior to initiating the conversion assay. The assay was performed in a plate-based format with each parasite isolate split over 3 wells (one for each media type) at 1% parasitemia in 5% hematocrit. Conversion rates were calculated by Giemsa-stained smears [25].

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Mosquito infections

Laboratory colonies of *An. stephensi* (Nijmegen Sind-Kasur strain)[38], *An. coluzzii* (N'gousso strain) [39] and *An. gambiae* s.s (Kisumu strain) [40], were maintained under controlled conditions: 26°C, 70-80% humidity and a 12-hour reverse day/night cycle. Groups of 100 female *Anopheles stephensi*, *An. gambiae*, and *An. coluzzii* mosquitoes, aged 1–5 days, were blood-fed for 15 minutes using glass membrane mini-feeders (15 mm diameter, convex bottom) connected to a heated circulating water bath. Fully fed mosquitoes were selected, and maintained at 30°C with access to 5-10% glucose.

Ookinetes count

Mosquito midguts were examined 18-24 hours post-infection to identify the presence of round forms, retort forms, and mature ookinetes. Five midguts from each infection group were dissected and incubated with a 1:50 diluted Anti-25KD-FITC conjugate in Evans blue solution. Midguts were gently disrupted using a pipette tip to release the blood meal. Following incubation in the dark for 30 minutes at room temperature, the solution was washed with 1.4 mL of phosphate-buffered saline (PBS), vortexed to dissolve the pellet, and centrifuged for 2 minutes at 10,000 rpm. After removal of the supernatant, the pellet was resuspended in 25 µL of PBS and 5 µL of the suspension was loaded into a Bürker-Turk counting chamber. Round forms, retort forms and mature ookinetes were counted using an incident light fluorescence microscope with a GFP filter at 400× magnification.

Oocysts count

Seven days post-infection, 20 mosquito midguts per group were dissected, stained with 1% mercurochrome, and examined under an optical microscope at 100× magnification to detect and quantify oocysts.

Sporozoites count

Mosquitoes used to examine sporozoite development received a second (uninfected) bloodmeal on day 4-6 post infection to synchronise oocyst development Fifteen days post-infection, mosquito salivary glands were dissected in PBS and transferred to oocyst lysis buffer (NaCl 0.1M: EDTA 25mM: TRIS-HCl 10mM). Following overnight incubation at 56°C with Proteinase K, DNA was extracted with the automated MagNA Pure LC instrument using the MagNA Pure LC DNA Isolation Kit – High performance. Sporozoite density was analysed using COX-I qPCR [41].

Statistical analysis

Statistical analyses were conducted using R software (v 3.1.12) [42]. Mean counts of oocysts, sporozoites, and ookinetes (with 95% confidence intervals) were estimated using a mixed-effects negative binomial regression model, incorporating random intercepts for biological replicates and

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

fixed effects for parasite line, mosquito species, and their interactions. Mean survival and commitment rates (with 95% confidence intervals) were calculated using a mixed-effects beta regression model. Transmission-reducing activity (TRA) and transmission-blocking activity (TBA)—reflecting reductions in oocyst density and the proportion of infected mosquitoes, respectively—were assessed for 700 nM and 7000 nM DHA using a Bayesian Poisson regression model.

Results

Parasite Backgrounds

We used well-characterised *P. falciparum* reference lines from Africa (NF54), Southeast Asia (NF135, Cambodia) [29], and a new isolate from East Africa (NF180, Uganda). These lines were complemented with previously published ART-R K13 mutant parasite lines from Thailand (ARN1G, K13-449A) [32], from Cambodia (3815, K13-580Y) [31, 35] and a recently collected isolate from Uganda with *in vitro* ART-R that is independent of K13 mutations (PAT-023). Genotyping revealed that some isolates harboured mutations associated with resistance to chloroquine and sulfadoxine-pyrimethamine (Table 1).

Artemisinin resistance

ART-R lines ARN1G and 3815 lines showed survival rates in the RSA that were higher than ART-S NF54, as previously published ([16, 32, 35];Figure 1a, Sup Table 1). The ART-S NF135 line showed variable survival rates, which were lower than those observed for known ART-R lines but higher than those observed for ART-S NF54 and NF180 lines. The PAT-023 parasite line exhibited a survival rate of 9.1% (95% CI: 6.6% - 12.5%), higher than the ART-S lines.

Sexual conversion

We assessed the ability of ART-R and ART-S lines to convert under two different conditions that mimic the natural sensing system [25, 43, 44]. All conversion assays were performed on a single asexual cycle. The highest rates of gametocyte conversion were typically observed using minimal fatty acid media with intermediate conversion rates for media containing Albumax only, and lowest conversion in non-inducing serum media (Figure 1b, Sup Table 2) [25]. The conversion rates observed for K13 mutants were comparable to those recorded for ART-S lines, except for the Cambodian line 3815, which exhibited highly variable conversion rates (Figure 1b). The Ugandan ART-R line PAT-023 exhibited minimal sexual conversion with little difference between media types (Figure 1b, Sup Table 2).

Transmission to mosquitoes

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Transmission was examined using three mosquito vector species. We first assessed whether fertilisation occurred in mosquitoes for each parasite isolate by quantifying parasite developmental stages in the mosquito midgut 20-24 hours post-infectious blood meal. We distinguished between mature and immature stages following Pfs25 antibody labelling. Immature stages included underdeveloped ookinetes and rounded forms classified as either zygotes or unfertilised females. The ART-S parasite isolates NF180 and NF135 showed no clear difference in the numbers of immature or mature ookinetes across the mosquito species tested (Figure 2a). For NF54, there was a tendency for fewer mature ookinetes in *An. gambiae* compared to *An. stephensi* and *An. coluzzii*, though this was not statistically significant (Figure 2a). A similar trend was observed with the ART-R ARN1G parasite line. For the Ugandan ART-R isolate PAT-023, significantly fewer mature ookinetes were observed in *An. stephensi* (Figure 2a) compared to *An. coluzzii* ($p = 0.019$). However, this significance level should be interpreted cautiously due to the number of comparisons made. The ART-R isolate 3815 consistently produced low numbers of mature ookinetes in all *Anopheles* species tested (Fig 2a).

To confirm that mature ookinetes can establish mosquito infections, we quantified oocyst prevalence (Sup Figure 1) and density (Figure 2b) on day 7 post-bloodmeal. African parasite lines (NF54, NF180, and PAT-023) exhibited higher oocyst densities in traditional African mosquitoes (*An. gambiae s.s., An. coluzzii*) compared to *An. stephensi,* particularly for PAT-023 ($p < 0.0001$; Sup Table 3). In contrast, NF135 showed consistent infection intensities across all tested mosquito species. For the Asian K13 mutant lines, oocysts densities in *An. stephensi* were slightly higher compared to *An. coluzzii* and *An. gambiae*. Specifically, K13 mutant parasite lines ARN1G had higher densities in *An. stephensi* relative to *An. coluzzii* ($p = 0.009$) and *An. gambiae* ($p < 0.0001$), while 3815 showed similar trends ($p = 0.007$ compared to *An. coluzzii* and $p<0.0001$ compared to *An. gambiae*; Sup Table 3). The 3815 isolate exhibited low and highly variable infection levels, consistent with its reduced mature ookinete counts (Figures 2a and 2b). Interestingly, a trend towards higher sporozoite production was observed in the two K13 mutant lines (ARN1G and 3815), which persisted even after normalising for oocyst density within the same batch of mosquitoes. However, transmission results for the 3815 isolate remained highly variable (Sup Figure 2).

Transmission-blocking effect of DHA

With all parasite lines being able to infect mosquitoes in the absence of drug pressure, we determined the transmission-reducing effect of DHA on mosquito infection intensity and prevalence. Mature gametocytes were exposed to both the physiologically relevant concentration of 700nM and a tenfold higher concentration of 7000nM for 48 hours before mosquito feeding with the drug not removed before mosquito feeding [32, 45]. Given the labour-intensive nature of these assays and the

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

known permissiveness of *An. coluzzii* for the parasites tested herein, experiments were performed exclusively with this mosquito species. All isolates showed a consistent decrease in mosquito infection intensity and prevalence as DHA concentrations increased, independent of the K13 genotype and the parasite background (Figure 3, Sup Figure 3). At 7000nM DHA, sporadic infections were observed for the African isolates NF54, NF180, and PAT-023, but none of the Asian isolates infected mosquitoes at this concentration. When relative reductions in oocyst intensity were calculated, DHA exposure at 7000nM reduced oocyst intensities by >93% for all parasite isolates. There were no indications of reduced transmission-blocking efficacy of DHA for ART-R isolates.

Correlating resistance and transmission stages

Although the number of parasite isolates examined was modest, our data on asexual parasite survival under DHA pressure, combined with findings on sexual conversion and mosquito transmission, provide an opportunity to investigate whether ART-R confers a transmission advantage. We initially hypothesised that parasites with high levels of *in vitro* resistance to ART at the ring stage would show enhanced transmission to mosquitoes under drug pressure. However, no correlation was observed between parasite survival in the RSA and the reduction in oocyst density under DHA pressure (Figure 4a). In contrast with our initial hypothesis, we observed a weak negative correlation between parasite survival rates in the RSA and gametocyte conversion rates upon induction (Figure 4b).

Discussion

Understanding the transmission fitness of parasites that survive ART-based treatment is important for developing strategies that aim to prevent or slow down the spread of resistance. Some reports have suggested that K13 mutant parasites may have higher intrinsic gametocyte production [4, 46], increased survival of specifically male gametocytes under DHA exposure, and larger oocysts in the mosquito midgut [32].

We selected three parasite isolates from Southeast Asia and three from Sub-Saharan Africa, each with distinct K13 genotypes and genetic backgrounds. The Asian ART-R lines ARN1G and 3815 exhibited higher parasite survival in the RSA, consistent with previous studies [16, 32, 35]. PAT-023, a newly characterised Ugandan ART-R line that is wild-type for K13, demonstrated a high level of survival in the *in vitro* RSA. Interestingly, we also observed that NF135, a line previously associated with treatment failure following artemether-lumefantrine therapy *in vivo* [47], showed erratic and occasionally increased DHA survival rates.

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

This study focuses on the transmission potential of *P. falciparum* isolates, beginning with their commitment to sexual stages. Fatty acids play a key role in signalling the parasite to commit to sexual conversion [36, 37]; we used two methods targeting this signalling pathway to robustly compare sexual conversion rates. From all media conditions tested, minimal fatty acid media consistently produced the highest sexual conversion rates across all lines. Among the two ART-S parasite isolates, NF54 and NF135 consistently exhibited high conversion levels. The newly introduced NF180 line, which has not been characterised in detail previously, demonstrated high conversion rates, comparable to NF54 and higher than NF135. Among the ART-R parasite lines, PAT-023 displayed the lowest commitment to gametocyte production, while ARN1G showed commitment levels similar to NF135. The Cambodian 3815 line exhibited an inconsistent commitment to gametocyte production, with occasional high levels that decreased with prolonged culture, potentially reflecting epigenetic silencing during *in vitro* cultivation.

We observed no evidence of increased of sexual conversion in the ART-R isolates. While sexual conversion is a critical step for transmission, high commitment rates do not always correlate with high mosquito infectivity [25, 29]. The presence of mature ookinetes is a direct indicator of fertilisation in the mosquito environment. In our study, we fed the same gametocyte material to the three mosquito species. We reproducibly observed low transmission potential of the 3815 line, which consistently formed very few ookinetes in all species and exhibited low and sporadic oocyst infections. We also observed a tendency for African parasites to fare better in the African mosquitoes, illustrated by both the numbers of mature ookinetes and oocysts densities. Although our sample size is too small for a comprehensive analysis of the underlying biological mechanisms, one could speculate that genes influencing the parasite's ability to evade the mosquito immune system may play a role. Pfs47 is essential for *P. falciparum* infection in *An. gambiae* but not for *An. stephensi* [48, 49]. Contrary to this, all three Southeast Asian parasite lines were able to infect African mosquito vectors. Interestingly ARN1G achieved slightly higher ookinete numbers in *An. coluzzii* but this did not translate into higher oocyst densities. Similar to our observations on sexual conversion rates, we found no significant transmission advantage for ART-R parasite lines with reduced ART susceptibility in the RSA.

To confirm the ability of the parasites to complete sporogony, we quantified sporozoite numbers in mosquito salivary glands. Previous studies have suggested that K13 mutant lines might produce larger oocysts [32], potentially indicating increased sporozoite production. While we did not observe a clear increase in oocyst size (data not shown), sporozoite production varied between parasite lines. The two Asian ART-R lines, each with a unique K13 mutation, exhibited significantly higher sporozoite loads in at least one African mosquito species. This effect was most pronounced for ARN1G that

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

showed significantly higher sporozoite numbers in *An. coluzzii*, even after adjusting for oocyst density within the same mosquito batch. Further studies are needed to confirm these findings and to explore the underlying biological mechanisms. However, our results are consistent with the suggestion that the K13 mutation may lead to larger oocysts [32], which could lead to increased sporozoite production.

Lastly, we examined the impact of DHA exposure on transmission efficiency, hypothesising that parasite lines with increased survival rate of asexual parasites under DHA exposure may show similar survival advantages when gametocytes are exposed. Previous work has suggested that male gametocytes with K13 mutations may have partial protection against DHA [32, 50]. Using *An. coluzzii,* we tested two DHA concentrations and found no evidence that DHA was less effective against mature gametocytes and subsequently reducing transmission in ART-R parasites. Immature gametocytes are known to be more susceptible to artemisinins than mature gametocytes [32, 51]. Since we did not examine the impact of DHA on immature gametocytes, we cannot rule out preferential survival of immature ART-R gametocytes. Even at the highest DHA concentrations, both ART-S and ART-R African parasite lines occasionally infected mosquitoes. This observation is consistent with previous *ex vivo* experiments [45] and aligns with clinical studies showing that treatment with DHA-piperaquine fails to fully prevent transmission in the first weeks after treatment [52].

In this study, we examined a limited number of ART-S and ART-R parasite lines across three mosquito species. We observed no consistent evidence of increased gametocyte production or increased mosquito infectivity. While the transmission-reducing effect of DHA was imperfect, its efficacy was not reduced in gametocyte-producing lines with partial resistance to ARTs. To fully uncover the discrete impacts of individual K13 mutations on gametocyte production, infectivity and gametocyte resistance to ARTs, future studies using genetically engineered lines in controlled isogenic backgrounds are required.

Ethics declarations

Experiments with *in vitro* cultured parasites and *Anopheles* mosquitoes at Radboud University Medical Center were conducted following approval from the Radboud University Experimental Animal Ethical Committee (RUDEC 2009-019, RUDEC 2009-225).

Acknowledgements and financial support

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

We would like to thank Jolanda Klaassen, Laura Pelser-Posthumus, Astrid Pouwelsen and Jacqueline Kuhnen for breeding of mosquitoes and handling of the infected mosquitoes. We are grateful for the support of Marga de Vegte-Bolmer in starting parasite culture with the 3815 line. DAF gratefully acknowledges funding support from the NIH (R01 AI109023). SM acknowledges the support from the Human Frontier Science Program Long-term Fellowship (LT000976/2016-L) and funding from United States National Institutes of Health NIAID grant R01 AI182318 (PI: SM). This work was supported by a fellowship from the Netherlands Organization for Scientific Research (Vici fellowship NWO 09150182210039).

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

References

1. World Health Organization, *World malaria report.* 2024
2. Noedl, H., Y. Se, K. Schaecher, B.L. Smith, D. Socheat, M.M. Fukuda, and C. Artemisinin Resistance in Cambodia 1 Study, *Evidence of artemisinin-resistant malaria in western Cambodia.* N Engl J Med, 2008. **359**(24): p. 2619-20.10.1056/NEJMc0805011.
3. Dondorp, A.M., F. Nosten, P. Yi, D. Das, A.P. Phyo, J. Tarning, et al., *Artemisinin resistance in Plasmodium falciparum malaria.* N Engl J Med, 2009. **361**(5): p. 455-67.10.1056/NEJMoa0808859.
4. Amaratunga, C., P. Lim, S. Suon, S. Sreng, S. Mao, C. Sopha, et al., *Dihydroartemisinin-piperaquine resistance in Plasmodium falciparum malaria in Cambodia: a multisite prospective cohort study.* Lancet Infect Dis, 2016. **16**(3): p. 357-65.10.1016/S1473-3099(15)00487-9.
5. Mairet-Khedim, M., R. Leang, C. Marmai, N. Khim, S. Kim, S. Ke, et al., *Clinical and In Vitro Resistance of Plasmodium falciparum to Artesunate-Amodiaquine in Cambodia.* Clin Infect Dis, 2021. **73**(3): p. 406-413.10.1093/cid/ciaa628.
6. Phyo, A.P., E.A. Ashley, T.J.C. Anderson, Z. Bozdech, V.I. Carrara, K. Sriprawat, et al., *Declining Efficacy of Artemisinin Combination Therapy Against P. Falciparum Malaria on the Thai-Myanmar Border (2003-2013): The Role of Parasite Genetic Factors.* Clin Infect Dis, 2016. **63**(6): p. 784-791.10.1093/cid/ciw388.
7. Ashley, E.A., M. Dhorda, R.M. Fairhurst, C. Amaratunga, P. Lim, S. Suon, et al., *Spread of artemisinin resistance in Plasmodium falciparum malaria.* N Engl J Med, 2014. **371**(5): p. 411-23.10.1056/NEJMoa1314981.
8. Birnbaum, J., S. Scharf, S. Schmidt, E. Jonscher, W.A.M. Hoeijmakers, S. Flemming, et al., *A Kelch13-defined endocytosis pathway mediates artemisinin resistance in malaria parasites.* Science, 2020. **367**(6473): p. 51-59.10.1126/science.aax4735.
9. Miotto, O., R. Amato, E.A. Ashley, B. MacInnis, J. Almagro-Garcia, C. Amaratunga, et al., *Genetic architecture of artemisinin-resistant Plasmodium falciparum.* Nat Genet, 2015. **47**(3): p. 226-34.10.1038/ng.3189.
10. Straimer, J., N.F. Gnadig, B. Witkowski, C. Amaratunga, V. Duru, A.P. Ramadani, et al., *Drug resistance. K13-propeller mutations confer artemisinin resistance in Plasmodium falciparum clinical isolates.* Science, 2015. **347**(6220): p. 428-31.10.1126/science.1260867.
11. Bridgford, J.L., S.C. Xie, S.A. Cobbold, C.F.A. Pasaje, S. Herrmann, T. Yang, et al., *Artemisinin kills malaria parasites by damaging proteins and inhibiting the proteasome.* Nat Commun, 2018. **9**(1): p. 3801.10.1038/s41467-018-06221-1.
12. Duffy, S. and V.M. Avery, *Naturally Acquired Kelch13 Mutations in Plasmodium falciparum Strains Modulate In Vitro Ring-Stage Artemisinin-Based Drug Tolerance and Parasite Survival in Response to Hyperoxia.* Microbiol Spectr, 2022. **10**(5): p. e0128221.10.1128/spectrum.01282-21.
13. Behrens, H.M., S. Schmidt, I.G. Henshall, P. Lopez-Barona, D. Peigney, R. Sabitzki, et al., *Impact of different mutations on Kelch13 protein levels, ART resistance, and fitness cost in Plasmodium falciparum parasites.* mBio, 2024. **15**(6): p. e0198123.10.1128/mbio.01981-23.
14. Nair, S., X. Li, G.A. Arya, M. McDew-White, M. Ferrari, F. Nosten, and T.J.C. Anderson, *Fitness Costs and the Rapid Spread of kelch13-C580Y Substitutions Conferring Artemisinin Resistance.* Antimicrob Agents Chemother, 2018. **62**(9).10.1128/AAC.00605-18.
15. Mok, S., B.H. Stokes, N.F. Gnadig, L.S. Ross, T. Yeo, C. Amaratunga, et al., *Artemisinin-resistant K13 mutations rewire Plasmodium falciparum's intra-erythrocytic metabolic program to enhance survival.* Nat Commun, 2021. **12**(1): p. 530.10.1038/s41467-020-20805-w.
16. Stokes, B.H., S.K. Dhingra, K. Rubiano, S. Mok, J. Straimer, N.F. Gnadig, et al., *Plasmodium falciparum K13 mutations in Africa and Asia impact artemisinin resistance and parasite fitness.* Elife, 2021. **10**.10.7554/eLife.66277.

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

17. Ariey, F., B. Witkowski, C. Amaratunga, J. Beghain, A.C. Langlois, N. Khim, et al., *A molecular marker of artemisinin-resistant Plasmodium falciparum malaria.* Nature, 2014. **505**(7481): p. 50-5.10.1038/nature12876.
18. Menard, D., N. Khim, J. Beghain, A.A. Adegnika, M. Shafiul-Alam, O. Amodu, et al., *A Worldwide Map of Plasmodium falciparum K13-Propeller Polymorphisms.* N Engl J Med, 2016. **374**(25): p. 2453-64.10.1056/NEJMoa1513137.
19. Conrad, M.D., V. Asua, S. Garg, D. Giesbrecht, K. Niare, S. Smith, et al., *Evolution of Partial Resistance to Artemisinins in Malaria Parasites in Uganda.* N Engl J Med, 2023. **389**(8): p. 722-732.10.1056/NEJMoa2211803.
20. Grossman, T., J. Vainer, Y. Paran, L. Studentsky, U. Manor, R. Dzikowski, and E. Schwartz, *Emergence of artemisinin-based combination treatment failure in patients returning from sub-Saharan Africa with P. falciparum malaria.* J Travel Med, 2023. **30**(8).10.1093/jtm/taad114.
21. Uwimana, A., E. Legrand, B.H. Stokes, J.M. Ndikumana, M. Warsame, N. Umulisa, et al., *Emergence and clonal expansion of in vitro artemisinin-resistant Plasmodium falciparum kelch13 R561H mutant parasites in Rwanda.* Nat Med, 2020. **26**(10): p. 1602-1608.10.1038/s41591-020-1005-2.
22. Rosenthal, P.J., V. Asua, J.A. Bailey, M.D. Conrad, D.S. Ishengoma, M.R. Kamya, et al., *The emergence of artemisinin partial resistance in Africa: how do we respond?* Lancet Infect Dis, 2024. **24**(9): p. e591-e600.10.1016/S1473-3099(24)00141-5.
23. Rosenthal, P.J., V. Asua, and M.D. Conrad, *Emergence, transmission dynamics and mechanisms of artemisinin partial resistance in malaria parasites in Africa.* Nat Rev Microbiol, 2024. **22**(6): p. 373-384.10.1038/s41579-024-01008-2.
24. Stewart, L.B., A. Freville, T.S. Voss, D.A. Baker, G.A. Awandare, and D.J. Conway, *Plasmodium falciparum Sexual Commitment Rate Variation among Clinical Isolates and Diverse Laboratory-Adapted Lines.* Microbiol Spectr, 2022. **10**(6): p. e0223422.10.1128/spectrum.02234-22.
25. Graumans, W., A. van der Starre, R. Stoter, G.J. van Gemert, C. Andolina, J. Ramjith, et al., *AlbuMAX supplemented media induces the formation of transmission-competent P. falciparum gametocytes.* Mol Biochem Parasitol, 2024. **259**: p. 111634.10.1016/j.molbiopara.2024.111634.
26. Cohuet, A., C. Harris, V. Robert, and D. Fontenille, *Evolutionary forces on Anopheles: what makes a malaria vector?* Trends Parasitol, 2010. **26**(3): p. 130-6.10.1016/j.pt.2009.12.001.
27. Ashine, T., A. Eyasu, Y. Asmamaw, E. Simma, E. Zemene, A. Epstein, et al., *Spatiotemporal distribution and bionomics of Anopheles stephensi in different eco-epidemiological settings in Ethiopia.* Parasit Vectors, 2024. **17**(1): p. 166.10.1186/s13071-024-06243-3.
28. Emiru, T., D. Getachew, M. Murphy, L. Sedda, L.A. Ejigu, M.G. Bulto, et al., *Evidence for a role of Anopheles stephensi in the spread of drug- and diagnosis-resistant malaria in Africa.* Nat Med, 2023. **29**(12): p. 3203-3211.10.1038/s41591-023-02641-9.
29. van de Vegte-Bolmer, M., W. Graumans, R. Stoter, G.J. van Gemert, R. Sauerwein, K.A. Collins, and T. Bousema, *A portfolio of geographically distinct laboratory-adapted Plasmodium falciparum clones with consistent infection rates in Anopheles mosquitoes.* Malar J, 2021. **20**(1): p. 381.10.1186/s12936-021-03912-x.
30. Teirlinck, A.C., M. Roestenberg, M. van de Vegte-Bolmer, A. Scholzen, M.J. Heinrichs, R. Siebelink-Stoter, et al., *NF135.C10: a new Plasmodium falciparum clone for controlled human malaria infections.* J Infect Dis, 2013. **207**(4): p. 656-60.10.1093/infdis/jis725.
31. Imwong, M., K. Suwannasin, C. Kunasol, K. Sutawong, M. Mayxay, H. Rekol, et al., *The spread of artemisinin-resistant Plasmodium falciparum in the Greater Mekong subregion: a molecular epidemiology observational study.* Lancet Infect Dis, 2017. **17**(5): p. 491-497.10.1016/S1473-3099(17)30048-8.

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

32. Witmer, K., F.A. Dahalan, M.J. Delves, S. Yahiya, O.J. Watson, U. Straschil, et al., *Transmission of Artemisinin-Resistant Malaria Parasites to Mosquitoes under Antimalarial Drug Pressure.* Antimicrob Agents Chemother, 2020. **65**(1).10.1128/AAC.00898-20.
33. Ponnudurai, T., A.H. Lensen, A.D. Leeuwenberg, and J.H. Meuwissen, *Cultivation of fertile Plasmodium falciparum gametocytes in semi-automated systems. 1. Static cultures.* Trans R Soc Trop Med Hyg, 1982. **76**(6): p. 812-8.10.1016/0035-9203(82)90116-x.
34. Phelan, J.E., A. Turkiewicz, E. Manko, J. Thorpe, L.N. Vanheer, M. van de Vegte-Bolmer, et al., *Rapid profiling of Plasmodium parasites from genome sequences to assist malaria control.* Genome Med, 2023. **15**(1): p. 96.10.1186/s13073-023-01247-7.
35. Witkowski, B., N. Khim, P. Chim, S. Kim, S. Ke, N. Kloeung, et al., *Reduced artemisinin susceptibility of Plasmodium falciparum ring stages in western Cambodia.* Antimicrob Agents Chemother, 2013. **57**(2): p. 914-23.10.1128/AAC.01868-12.
36. Harris, C.T., X. Tong, R. Campelo, I.M. Marreiros, L.N. Vanheer, N. Nahiyaan, et al., *Sexual differentiation in human malaria parasites is regulated by competition between phospholipid metabolism and histone methylation.* Nat Microbiol, 2023. **8**(7): p. 1280-1292.10.1038/s41564-023-01396-w.
37. Brancucci, N.M.B., J.P. Gerdt, C. Wang, M. De Niz, N. Philip, S.R. Adapa, et al., *Lysophosphatidylcholine Regulates Sexual Stage Differentiation in the Human Malaria Parasite Plasmodium falciparum.* Cell, 2017. **171**(7): p. 1532-1544 e15.10.1016/j.cell.2017.10.020.
38. Feldmann, A.M. and T. Ponnudurai, *Selection of Anopheles stephensi for refractoriness and susceptibility to Plasmodium falciparum.* Med Vet Entomol, 1989. **3**(1): p. 41-52.10.1111/j.1365-2915.1989.tb00473.x.
39. Meister, S., B. Agianian, F. Turlure, A. Relogio, I. Morlais, F.C. Kafatos, and G.K. Christophides, *Anopheles gambiae PGRPLC-mediated defense against bacteria modulates infections with malaria parasites.* PLoS Pathog, 2009. **5**(8): p. e1000542.10.1371/journal.ppat.1000542.
40. Deletre, E., T. Martin, C. Dumenil, and F. Chandre, *Insecticide resistance modifies mosquito response to DEET and natural repellents.* Parasit Vectors, 2019. **12**(1): p. 89.10.1186/s13071-019-3343-9.
41. Andolina, C., W. Graumans, M. Guelbeogo, G.J. van Gemert, J. Ramijth, S. Harouna, et al., *Quantification of sporozoite expelling by Anopheles mosquitoes infected with laboratory and naturally circulating P. falciparum gametocytes.* Elife, 2024. **12**.10.7554/eLife.90989.
42. RC, T. *The R project for statistical computing Cran.* 2019; Available from: https://www.r-project.org/.
43. Omorou, R., I. Bin Sa'id, M. Delves, C. Severini, Y.I. Kouakou, A.L. Bienvenu, and S. Picot, *Protocols for Plasmodium gametocyte production in vitro: an integrative review and analysis.* Parasit Vectors, 2022. **15**(1): p. 451.10.1186/s13071-022-05566-3.
44. Tanaka, T.Q., S.M. Tokuoka, D. Nakatani, F. Hamano, S.I. Kawazu, T.E. Wellems, et al., *Polyunsaturated fatty acids promote Plasmodium falciparum gametocytogenesis.* Biol Open, 2019. **8**(7).10.1242/bio.042259.
45. Henry, N.B., I. Soulama, S.S. S, J.M. Bolscher, T.G.H. T, S.C. A, et al., *Assessment of the transmission blocking activity of antimalarial compounds by membrane feeding assays using natural Plasmodium falciparum gametocyte isolates from West-Africa.* PLoS One, 2023. **18**(7): p. e0284751.10.1371/journal.pone.0284751.
46. Zhu, L., R.W. van der Pluijm, M. Kucharski, S. Nayak, J. Tripathi, N.J. White, et al., *Artemisinin resistance in the malaria parasite, Plasmodium falciparum, originates from its initial transcriptional response.* Commun Biol, 2022. **5**(1): p. 274.10.1038/s42003-022-03215-0.
47. van der Boor, S.C., M. Alkema, G.J. van Gemert, K. Teelen, M. van de Vegte-Bolmer, J. Walk, et al., *Whole sporozoite immunization with Plasmodium falciparum strain NF135 in a randomized trial.* BMC Med, 2023. **21**(1): p. 137.10.1186/s12916-023-02788-9.

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

48. Molina-Cruz, A., L.S. Garver, A. Alabaster, L. Bangiolo, A. Haile, J. Winikor, et al., *The human malaria parasite Pfs47 gene mediates evasion of the mosquito immune system.* Science, 2013. **340**(6135): p. 984-7.10.1126/science.1235264.

49. van Schaijk, B.C., M.R. van Dijk, M. van de Vegte-Bolmer, G.J. van Gemert, M.W. van Dooren, S. Eksi, et al., *Pfs47, paralog of the male fertility factor Pfs48/45, is a female specific surface protein in Plasmodium falciparum.* Mol Biochem Parasitol, 2006. **149**(2): p. 216-22.10.1016/j.molbiopara.2006.05.015.

50. Lozano, S., P. Gamallo, C. Gonzalez-Cortes, J.L. Presa Matilla, R.M. Fairhurst, E. Herreros, et al., *Gametocytes from K13 Propeller Mutant Plasmodium falciparum Clinical Isolates Demonstrate Reduced Susceptibility to Dihydroartemisinin in the Male Gamete Exflagellation Inhibition Assay*. Antimicrob Agents Chemother, 2018. **62**(12).10.1128/AAC.01426-18.

51. Adjalley, S.H., G.L. Johnston, T. Li, R.T. Eastman, E.H. Ekland, A.G. Eappen, et al., *Quantitative assessment of Plasmodium falciparum sexual development reveals potent transmission-blocking activity by methylene blue.* Proc Natl Acad Sci U S A, 2011. **108**(47): p. E1214-23.10.1073/pnas.1112037108.

52. Stone, W., A. Mahamar, K. Sanogo, Y. Sinaba, S.M. Niambele, A. Sacko, et al., *Pyronaridine-artesunate or dihydroartemisinin-piperaquine combined with single low-dose primaquine to prevent Plasmodium falciparum malaria transmission in Ouelessebougou, Mali: a four-arm, single-blind, phase 2/3, randomised trial.* Lancet Microbe, 2022. **3**(1): p. e41-e51.10.1016/S2666-5247(21)00192-0.

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Tables

Table 1. Parasite isolates

Parasite Isolate	Origin	Drug resistance-conferring mutations				
		Artemisinin	Chloroquine	Mefloquine	Pyrimethamine	Sulfadoxine
NF54	Africa	-	-	-	-	-
NF180	East Africa (Uganda)	-	-	-	DHFR (N51I, S108N)	DHPS (A437G, K540E)
PAT-023	East Africa (Uganda)	-	PfMDR1 (Y184F)	-	DHFR (C59R, S108N)	DHPS (A437G, K540E)
NF135	Southeast Asia (Cambodia)	-	PfCRT (M74I, N75E, K76T, A220S, Q271E, I356T, R371I)	-	DHFR (N51I, C59R, S108N, I164L)	DHPS (S436A, A437G, K540E)
ARN1G	Southeast Asia (Thailand)	K13 (G449A)	PfCRT (M74I, N75E, K76T, A220S, Q271E, N326S, I356T, R371I)	-	DHFR (N51I, C59R, S108N, I164L)	DHPS (A437G, K540E, A581G)
3815	Southeast Asia (Cambodia)	K13 (C580Y)	PfCRT (M74I, N75E, K76T, A220S, Q271E, I356T, R371I)	-	DHFR (N51I, C59R, S108N, I164L)	DHPS (S436A, A437G, K540E)

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Figure Legends

Figure 1. Parasite survival after exposure to dihydroartemisinin (DHA) and gametocyte conversion rates of *P. falciparum* isolates with different K13 genotypes and genetic backgrounds. **A)** Survival rate from the Ring-stage Survival Assay (RSA) of each parasite line following 6hr exposure to 700nM DHA compared to DMSO controls in duplicate wells (4-6 replicates were performed for each parasite line). The dots represent biological replicate survival rates at 72h post invasion from counts either by microscopy or flow cytometry and the error bars represent the 95% confidence intervals. **B)** Sexual conversion in a plate-based assay that quantifies conversion rates from a single asexual round using three different media types (shown different colour gradient of the bars). Conversion rates are calculated by dividing the final gametocytaemia by the starting parasitemia in the same well. Dots represent a single well from independent plates; error bars represent 95% confidence intervals.

Figure 2. Infection of three mosquito species with the six different parasite lines. **A)** Ookinete counts 20 hours after blood meal. The ookinetes were stained with anti-Pfs25 488 conjugate and counted on a haemocytometer under a fluorescent microscope. Immature forms included rounded zygotes (or unfertilised females) and ookinetes with incomplete maturation (lighter shading bar), while mature forms were only the completely mature ookinetes (dark shaded bar). Dots represent a single count from an independent experiment and the error bars represent 95% confidence intervals. **B)** Average oocysts per midgut were dissected on day 7 post-bloodmeal. A total of 20 mosquitoes per group were dissected and oocysts were counted by mercurochrome staining. Dots represent the counted oocysts from a single midgut; error bars represent 95% confidence intervals. **C)** Salivary glands from individual mosquitoes were dissected and sporozoites quantified by qPCR. The dots represent the sporozoites per salivary gland from a single mosquito and the error bars represent the confidence intervals.

Figure 3. Transmission reduction in the presence of DHA. **A)** Oocyst densities observed upon exposing mature gametocytes to 700nM and 7000nM DHA prior to being fed to mosquitoes in a blood meal. The dots represent the average oocysts per infected mosquito from a single cage. **B)** Relative reductions in oocysts density compared the no drug control. All error bars represent the confidence intervals.

Figure 4. Impact of resistance on transmission and commitment. Transmission **A)** and conversion **B)** plotted against survival rates from the RSA for each parasite line. The underlying detailed data on *in vitro* parasite resistance in the RSA and gametocyte commitment are presented in Figure 1; data on transmission under DHA exposure are presented in Figure 3.

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

523

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Supplemental Tables

Sup Table 1. RSA comparisons

Reference parasite line	Parasite line	Ratio (CI)
NF54	NF180	0.99 (0.25, 3.85); p=0.9742
NF54	PAT-023	33.34 (10, 100); p<0.0001
NF54	NF135	6.67 (1.49, 33.34); p=0.0023
NF54	ARN1G	20.00 (6.25, 100); p<0.0001
NF54	3815	33.34 (12.5, 100); p<0.0001
NF135	PAT-023	4.31 (1.20, 15.49); p=0.0057
NF135	ARN1G	3.13 (0.81, 12.5); p=0.0636
NF135	3815	5.26 (1.51, 20); p=0.0014

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

527 **Sup Table 2. Sexual conversion comparisons**

Parasite line	Reference media	Media	Ratio (CI)
NF54	serum	mFa	114.14 (31.57, 412.62); p<0.0001
NF54	serum	Albumax	57.67 (15.77, 210.87); p<0.0001
NF54	mFa	Albumax	0.51 (0.16, 1.60); p=0.1516
NF180	serum	mFa	46.96 (14.23, 154.93); p<0.0001
NF180	serum	Albumax	21.99 (6.47, 74.73); p<0.0001
NF180	mFa	Albumax	0.47 (0.16, 1.39); p=0.0934
PAT-023	serum	mFa	3.95 (1.06, 14.70); p=0.0374
PAT-023	serum	Albumax	2.90 (0.78, 10.79); p=0.1027
PAT-023	mFa	Albumax	0.73 (0.20, 2.74); p=0.5691
NF135	serum	mFa	92.75 (25.51, 337.27); p<0.0001
NF135	serum	Albumax	27.16 (6.95, 106.13); p<0.0001
NF135	mFa	Albumax	0.29 (0.09, 1.01); p=0.0172
ARN1G	serum	mFa	75.74 (22.86, 250.88); p<0.0001
ARN1G	serum	Albumax	30.40 (8.75, 105.61); p<0.0001
ARN1G	mFa	Albumax	0.40 (0.13, 1.24); p=0.0511
3815	serum	mFa	7.27 (1.97, 26.84); p=0.0011
3815	serum	Albumax	4.30 (1.16, 16.00); p=0.0162
3815	mFa	Albumax	0.59 (0.16, 2.19); p=0.3311

528 mFa, minimal fatty acid.

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

529 **Sup Table 3. Oocyst comparisons**

Parasite line	Reference mosquito species	Mosquito species	Oocyst ratio
NF54	*An. stephensi*	*An. coluzzii*	4.35 (2.5, 7.69); p<0.0001
NF54	*An. stephensi*	*An. gambiae*	1.56 (0.87, 2.78); p=0.0695
NF54	*An. coluzzii*	*An. gambiae*	0.35 (0.20, 0.62); p<0.0001
NF180	*An. stephensi*	*An. coluzzii*	1.66 (1.01, 2.70); p=0.0285
NF180	*An. stephensi*	*An. gambiae*	2.17 (1.33, 3.57); p=0.0005
NF180	*An. coluzzii*	*An. gambiae*	1.31 (0.81, 2.12); p=0.1829
PAT-023	*An. stephensi*	*An. coluzzii*	6.25 (3.45, 11.11); p<0.0001
PAT-023	*An. stephensi*	*An. gambiae*	3.45 (1.96, 5.88); p<0.0001
PAT-023	*An. coluzzii*	*An. gambiae*	0.55 (0.32, 0.94); p=0.0079
NF135	*An. stephensi*	*An. coluzzii*	1.09 (0.66, 1.79); p=0.6706
NF135	*An. stephensi*	*An. gambiae*	0.69 (0.42, 1.12); p=0.1353
NF135	*An. coluzzii*	*An. gambiae*	0.63 (0.38, 1.03); p=0.0732
ARN1G	*An. stephensi*	*An. coluzzii*	0.53 (0.31, 0.9); p=0.0087
ARN1G	*An. stephensi*	*An. gambiae*	0.30 (0.18, 0.53); p<0.0001
ARN1G	*An. coluzzii*	*An. gambiae*	0.57 (0.32, 1.00); p=0.0167
3815	*An. stephensi*	*An. coluzzii*	0.44 (0.22, 0.9); p=0.0066
3815	*An. stephensi*	*An. gambiae*	0.08 (0.03, 0.24); p<0.0001
3815	*An. coluzzii*	*An. gambiae*	0.18 (0.06, 0.56); p=0.0006

530

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

531 **Sup Table 4. Sporozoite comparisons**

Parasite line	Reference mosquito species	Mosquito species	Ratio (CI)
NF54	*An. stephensi*	*An. coluzzii*	0.28 (0.04, 2.0); p=0.2362
NF54	*An. stephensi*	*An. gambiae*	0.11 (0.01, 0.79); p=0.0236
NF54	*An. coluzzii*	*An. gambiae*	0.38 (0.07, 2.02); p=0.2362
NF180	*An. stephensi*	*An. coluzzii*	1.30 (0.31, 5.26); p=0.6562
NF180	*An. stephensi*	*An. gambiae*	0.28 (0.07, 1.19); p=0.0702
NF180	*An. coluzzii*	*An. gambiae*	0.22 (0.05, 0.92); p=0.0333
PAT-023	*An. stephensi*	*An. coluzzii*	1.69 (0.17, 16.67); p=1.0000
PAT-023	*An. stephensi*	*An. gambiae*	1.59 (0.14, 16.67); p=1.0000
PAT-023	*An. coluzzii*	*An. gambiae*	0.93 (0.08, 10.51); p=1.0000
NF135	*An. stephensi*	*An. coluzzii*	0.09 (0.02, 0.46); p=0.0010
NF135	*An. stephensi*	*An. gambiae*	0.22 (0.04, 1.09); p=0.0488
NF135	*An. coluzzii*	*An. gambiae*	2.20 (0.48, 10.04); p=0.2114
ARN1G	*An. stephensi*	*An. coluzzii*	5.88 (1.35, 25.0); p=0.0084
ARN1G	*An. stephensi*	*An. gambiae*	0.35 (0.06, 1.92); p=0.1388
ARN1G	*An. coluzzii*	*An. gambiae*	0.06 (0.01, 0.29); p<0.0001
3815	*An. stephensi*	*An. coluzzii*	0.02 (0, 0.31); p=0.0011
3815	*An. stephensi*	*An. gambiae*	2.56 (0.2, 33.34); p=0.3758
3815	*An. coluzzii*	*An. gambiae*	106.97 (8.32, 1375.20); p<0.0001

532

533

bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

534 **Sup Table 5. Sporozoite per oocyst comparisons**

Parasite line	Reference mosquito species	Mosquito species	Sporozoite per oocyst ratio
NF54	*An. stephensi*	*An. coluzzii*	0.06 (0.01, 0.50); p=0.0044
NF54	*An. stephensi*	*An. gambiae*	0.07 (0.01, 0.56); p=0.0047
NF54	*An. gambiae*	*An. coluzzii*	0.92 (0.16, 5.33); p=0.9092
NF180	*An. stephensi*	*An. coluzzii*	0.78 (0.17, 3.51); p=0.6919
NF180	*An. stephensi*	*An. gambiae*	0.13 (0.03, 0.60); p=0.0042
NF180	*An. gambiae*	*An. coluzzii*	5.89 (1.32, 26.36); p=0.0096
PAT-023	*An. stephensi*	*An. coluzzii*	0.27 (0.03, 2.88); p=0.5525
PAT-023	*An. stephensi*	*An. gambiae*	0.46 (0.04, 5.59); p=0.9104
PAT-023	*An. gambiae*	*An. coluzzii*	0.59 (0.05, 7.06); p=0.9104
NF135	*An. stephensi*	*An. coluzzii*	0.09 (0.02, 0.45); p=0.0012
NF135	*An. stephensi*	*An. gambiae*	0.32 (0.06, 1.74); p=0.1198
NF135	*An. gambiae*	*An. coluzzii*	0.29 (0.06, 1.42); p=0.1198
ARN1G	*An. stephensi*	*An. coluzzii*	11.30 (2.31, 55.28); p=0.0009
ARN1G	*An. stephensi*	*An. gambiae*	1.15 (0.19, 6.97); p=0.8499
ARN1G	*An. gambiae*	*An. coluzzii*	9.81 (1.79, 53.69); p=0.0028
3815	*An. stephensi*	*An. coluzzii*	0.05 (0.00, 0.76); p=0.0084
3815	*An. stephensi*	*An. gambiae*	31.40 (1.95, 504.25); p=0.0062
3815	*An. gambiae*	*An. coluzzii*	0.00 (0.00, 0.03); p<0.0001

535

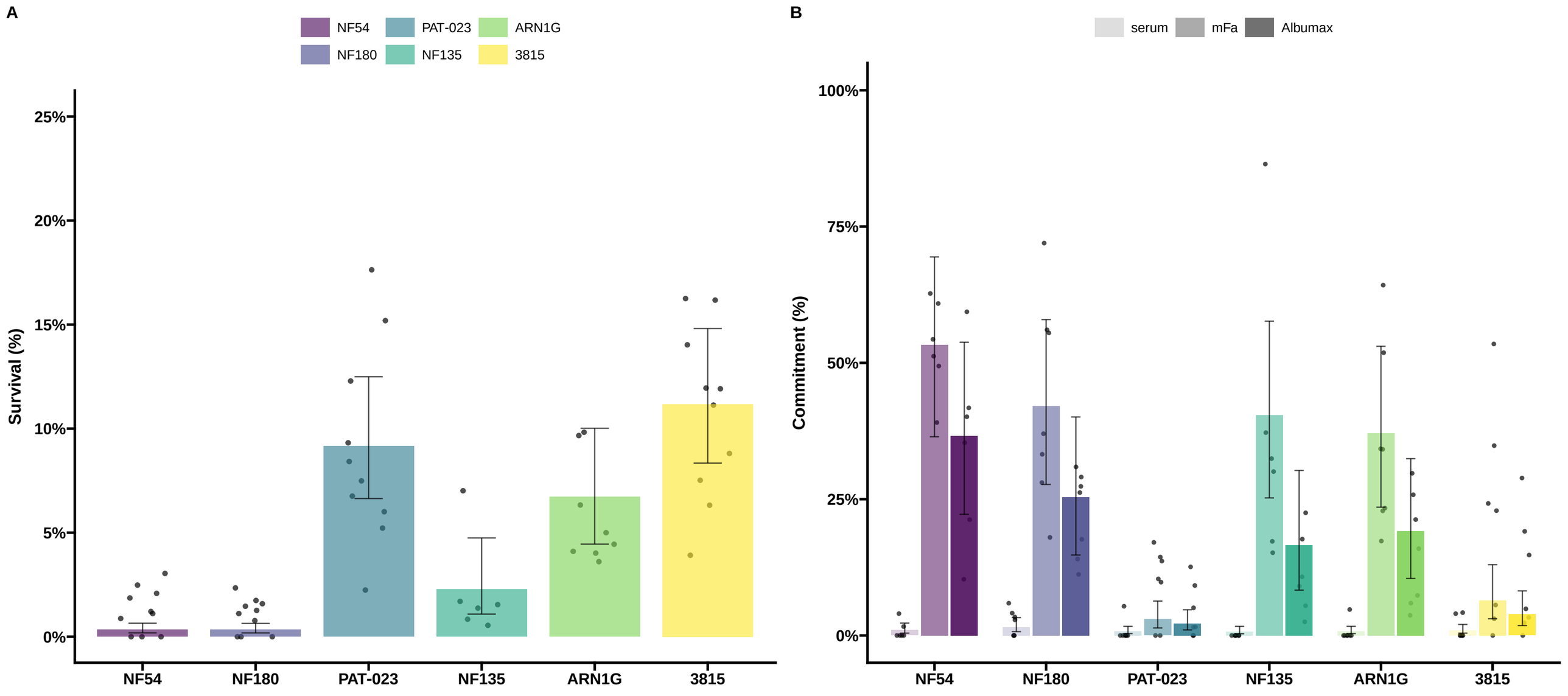
bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Supplemental Figure Legends

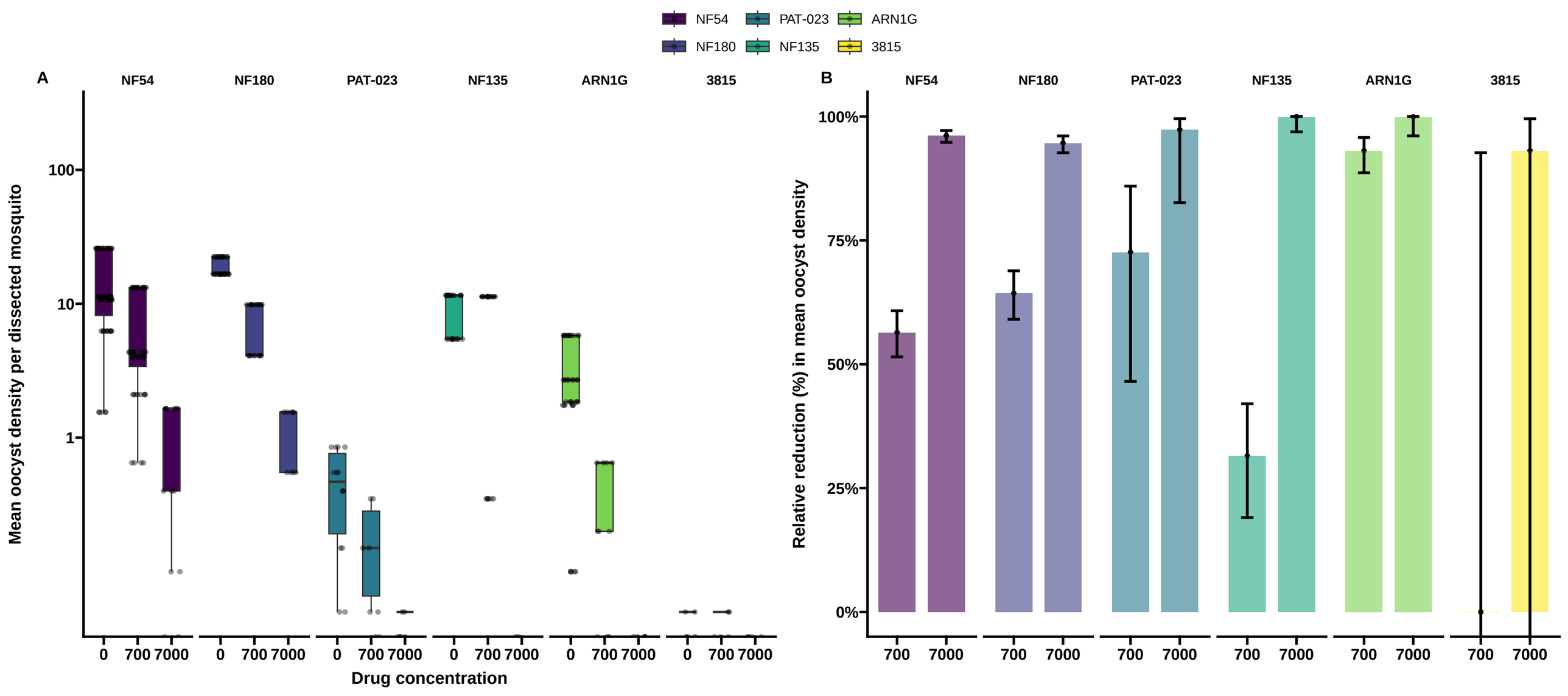
Sup Figure 1. Mosquito infection rates for the six parasite lines in each mosquito species across three to four independent mosquito feeding experiments.

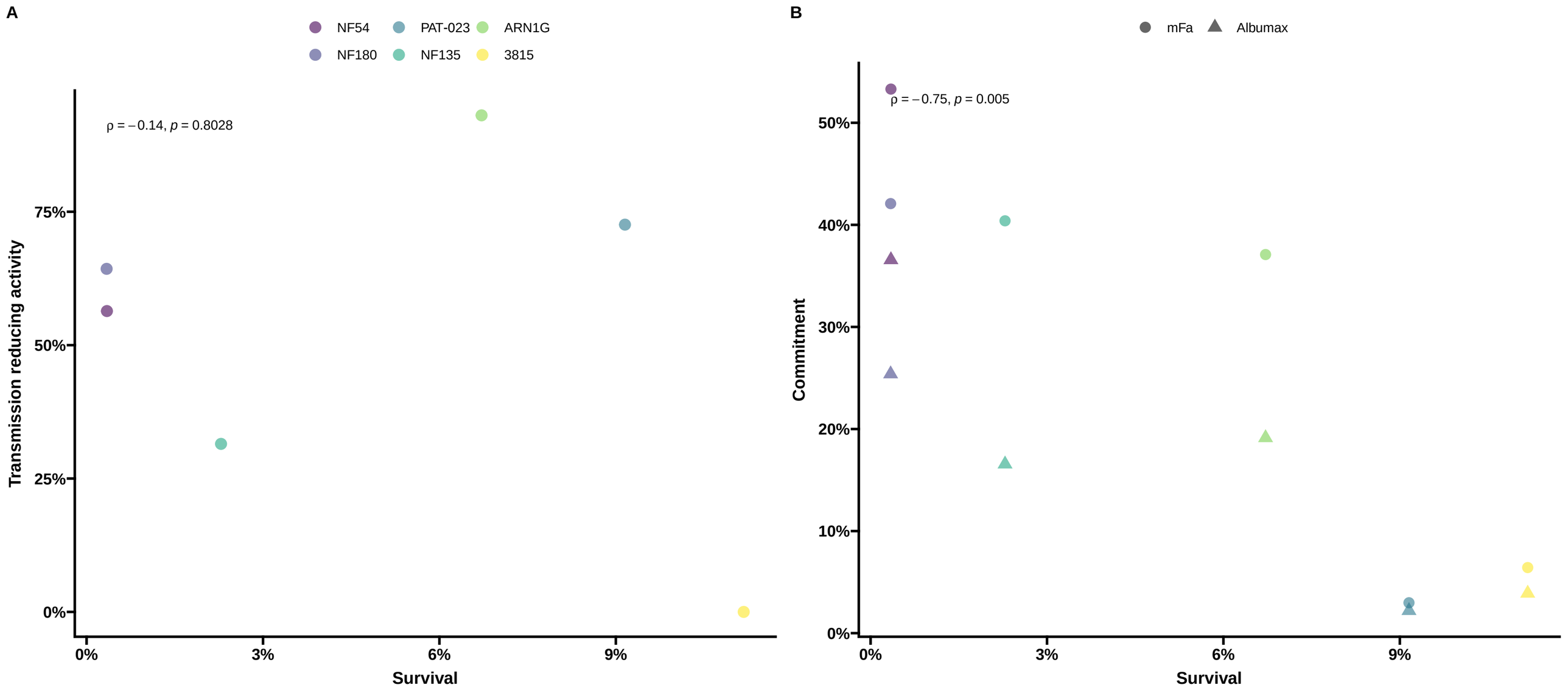
Sup Figure 2. Calculated sporozoite per oocyst**.** Sporozoites per salivary gland from individual mosquitoes were dissected and processed for qPCR. The sporozoites per oocysts was calculated based on the cage matched oocysts per mosquito average. The error bars represent the confidence intervals.

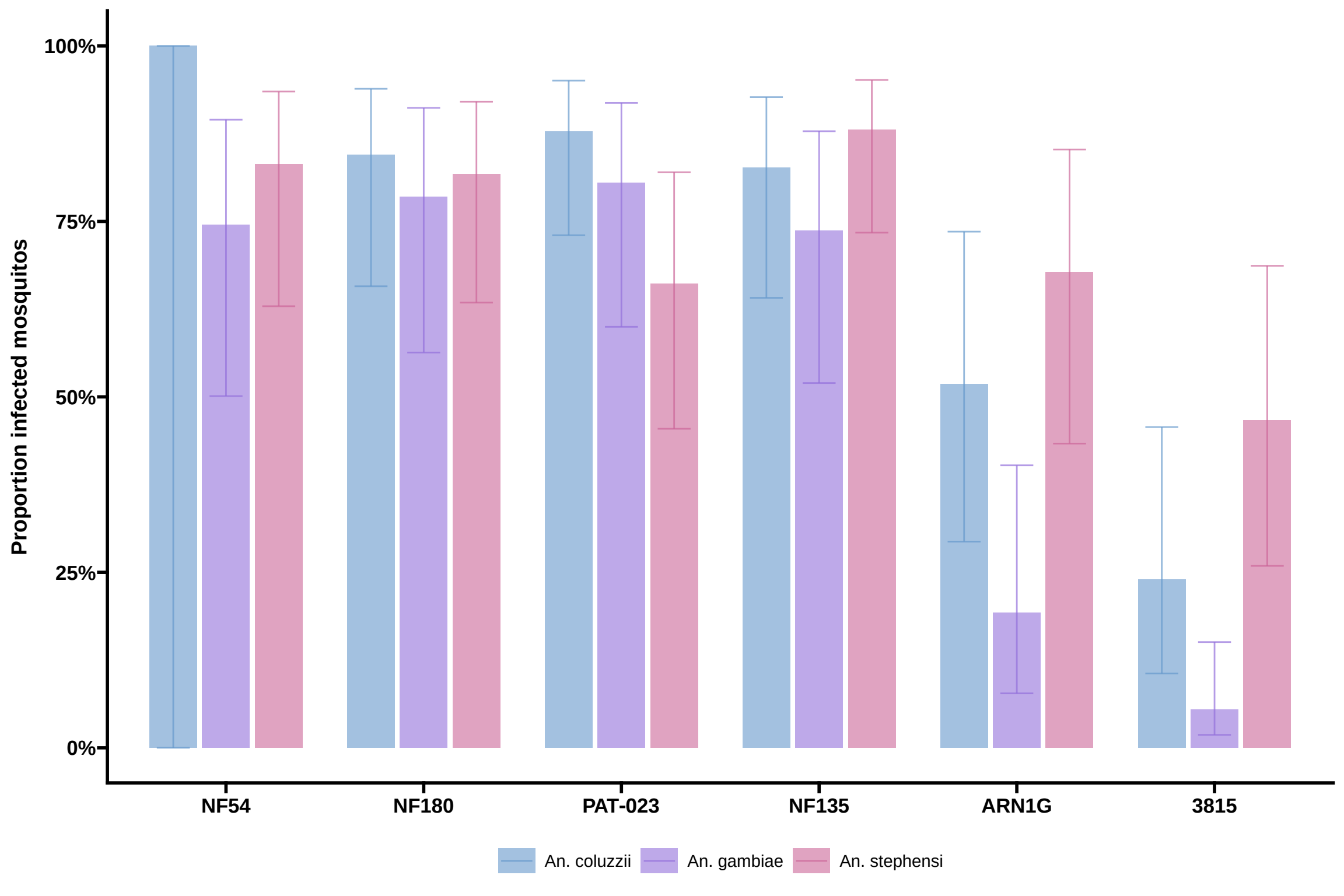
Sup Figure 3. Reduction in the overall oocyst prevalence of the six parasites lines following exposure to DHA. **A)** Shows the decrease in mosquito infectivity upon exposing mature gametocytes to 700nM and 7000nM DHA prior to being fed to mosquitoes in a blood meal. The dots represent the prevalence from an individual mosquito cage. **B)** Represents the relative reduction in oocysts density compared the no drug control. All error bars represent the confidence intervals.

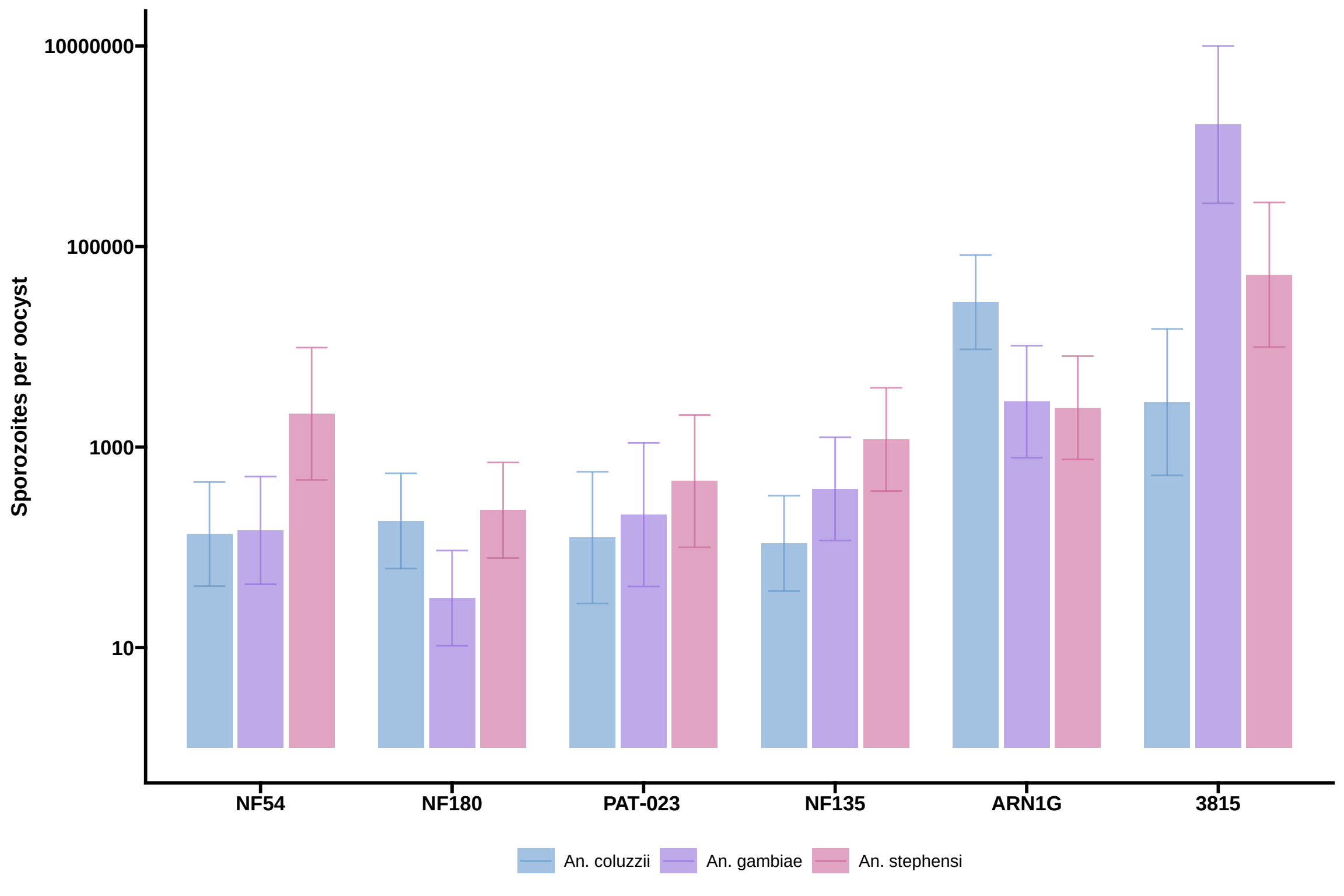

A
NF54
PAT-023
ARN1G
NF180
NF135
3815
Survival (%)
25%
20%
15%
10%
5%
0%
NF54
NF180
PAT-023
NF135
ARN1G
3815
B
serum
mFa
Albumax
Commitment (%)
100%
75%
50%
25%
0%
NF54
NF180
PAT-023
NF135
ARN1G
3815

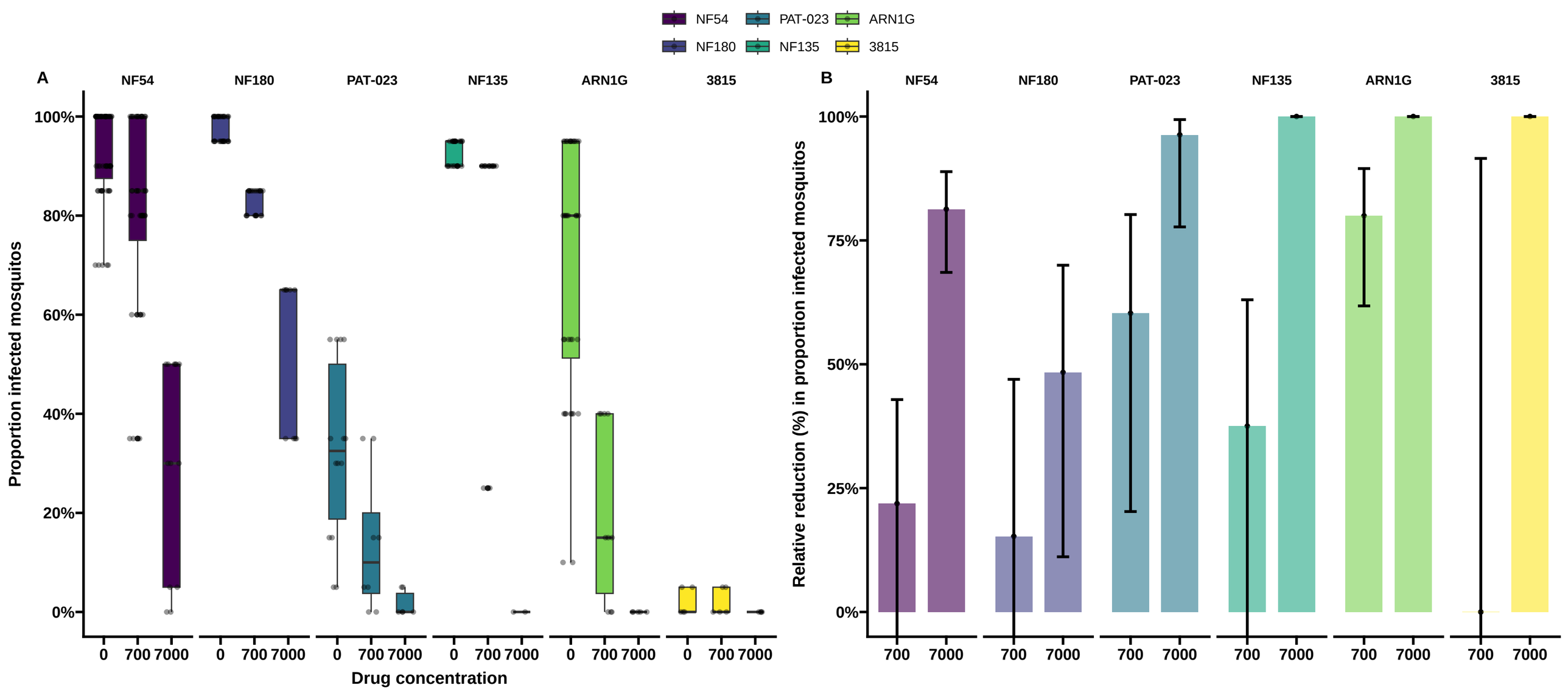
bioRxiv preprint doi: https://doi.org/10.1101/2024.12.23.630038; this version posted December 23, 2024. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.


NF54
PAT-023
ARN1G
NF180
NF135
3815
A
NF54
NF180
PAT-023
NF135
ARN1G
3815
Mean oocyst density per dissected mosquito
100
10
1
0
700
7000
Drug concentration
B
Relative reduction (%) in mean oocyst density
100%
75%
50%
25%
0%
700
7000


A
NF54
PAT-023
ARN1G
NF180
NF135
3815
ρ = −0.14, p = 0.8028
Transmission reducing activity
75%
50%
25%
0%
0%
3%
6%
9%
Survival
B
mFa
Albumax
ρ = −0.75, p = 0.005
Commitment
50%
40%
30%
20%
10%
0%
0%
3%
6%
9%
Survival


Proportion infected mosquitos
100%
75%
50%
25%
0%
NF54
NF180
PAT-023
NF135
ARN1G
3815
An. coluzzii
An. gambiae
An. stephensi


Sporozoites per oocyst
10000000
100000
1000
10
NF54
NF180
PAT-023
NF135
ARN1G
3815
An. coluzzii
An. gambiae
An. stephensi


NF54
PAT-023
ARN1G
NF180
NF135
3815
A
B
Proportion infected mosquitos
Relative reduction (%) in proportion infected mosquitos
Drug concentration
100%
80%
60%
40%
20%
0%
75%
50%
25%
0
700
7000