Check for updates

RESEARCH ARTICLE

Optimizing *Plasmodium vivax* gametocyte enrichment to enhance transmission to *Anopheles* mosquitoes

[version 1; peer review: 2 approved with reservations]

Tessa J.M Geraedts[1], Wakweya Chali[1,2], Geert-Jan van Gemert[1], Jordache Ramjith[1], Rianne Stoter[1], Kjerstin Lanke[1], Hester Vogelaar[1], Katherine A Collins[3], Nicholas Proellochs[1], Angela M. Minassian[4-7], Simon J. Draper[4-7], Fitsum Tadesse[1,2], Benjamin Mordmüller[1], Teun Bousema[1,8], Wouter Graumans[1]

[1]Radboudumc Afdeling Medische Microbiologie, Nijmegen, Gelderland, The Netherlands
[2]Armauer Hansen Research Institute, Addis Ababa, Addis Ababa, Ethiopia
[3]Open Philanthropy Project, San Francisco, California, USA
[4]NIHR Oxford Biomedical Research Centre, Oxford, England, UK
[5]Kavli Institute of Nanoscience, Oxford, UK
[6]University of Oxford Department of Paediatrics, Oxford, England, UK
[7]Department of Biochemistry, University of Oxford, Oxford, England, UK
[8]Immunology and Infection, London School of Hygiene & Tropical Medicine, London, England, UK

v1 **First published:** 22 Jul 2026, **6**:252
https://doi.org/10.12688/openreseurope.23635.1
Latest published: 22 Jul 2026, **6**:252
https://doi.org/10.12688/openreseurope.23635.1

Abstract

Background

Plasmodium vivax is the second leading cause of malaria worldwide. The lack of continuous *in vitro* culture of *P. vivax* hampers research efforts, including research on transmission to mosquitoes and sporozoite biology. Here, we present methodological improvements to achieve high mosquito infection rates.

Methods

We compared two gametocyte enrichment methods: magnetic cell sorting (MACS) and Percoll density gradient centrifugation (DGC) at 1500 *g* or 760 *g*. Blood samples before and after enrichment were offered to *Anopheles stephensi* mosquitoes by membrane feeding. Outcomes included the proportion of mosquitoes infected, oocyst density and sporozoite positivity. Protocols were first evaluated using *in vitro* cultured *P. falciparum*, before being further optimized using gametocyte-positive samples from eight healthy, malaria-naive adults

Open Peer Review

Approval Status ? ?

	1	2
version 1 22 Jul 2026	? view	? view

1. **Gustavo Capatti Cassiano**, Universidade NOVA De Lisboa (UNL), Lisboa, Portugal

2. **Prince Chigozirim Ubiaru**, University of Glasgow, Glasgow, UK
University of Nottingham, Nottingham, UK
Omar Janha, Advanced Research Centre, Glasgow, UK
Sabyasachi Pradhan, University of Glasgow College of Medical Veterinary and Life Sciences (Ringgold ID: 223860), Glasgow, UK

Any reports and responses or comments on the article can be found at the end of the article.

infected with a *P. vivax* clone 'PvW1' during a controlled human malaria infection.

Results

DGC at a speed of 1500 *g* achieved the highest infection rates. Oocyst density increased approximately 86-fold (95% CI 14–525; $p < 0.01$) and the proportion of infected mosquitoes was on average 58% higher when compared to the non-enriched control (95% CI 42.6–74.0; $p < 0.01$). DGC at 760 *g* increased the oocyst density 11-fold (CI 1.8–69; $p < 0.01$) and the proportion of infected mosquitoes by 28% (CI 12.1–43.9; $p < 0.01$). MACS did not increase *P. vivax* mosquito infection compared to control. Infected mosquitoes became salivary gland positive, with the mean sporozoite load in infected mosquitoes increasing with oocyst density.

Conclusions

This study provides the first systematic comparison of MACS and DGC as enrichment techniques to increase *P. vivax* transmission and presents the DGC protocol that achieved the highest proportion of infected mosquitoes and oocyst densities. This protocol will facilitate *P. vivax* transmission studies and enable the generation of highly infected mosquitoes and sporozoites for downstream use.

Keywords
Plasmodium vivax, Plasmodium falciparum, Controlled Human Malaria Infection, Percoll density gradient centrifugation, Magnetic cell sorting, Mosquito membrane feeding

HE This article is included in the Horizon Europe gateway.

Corresponding author: Wouter Graumans (wouter.graumans@radboudumc.nl)

Author roles: Geraedts TJM: Data Curation, Investigation, Validation, Writing – Original Draft Preparation, Writing – Review & Editing; **Chali W**: Writing – Review & Editing; **van Gemert GJ**: Investigation, Supervision, Writing – Review & Editing; **Ramjith J**: Data Curation, Formal Analysis, Validation, Visualization, Writing – Review & Editing; **Stoter R**: Investigation; **Lanke K**: Investigation, Writing – Review & Editing; **Vogelaar H**: Investigation, Writing – Review & Editing; **Collins KA**: Methodology, Writing – Review & Editing; **Proellochs N**: Writing – Review & Editing; **Minassian AM**: Funding Acquisition, Resources, Writing – Review & Editing; **Draper SJ**: Funding Acquisition, Resources, Writing – Review & Editing; **Tadesse F**: Supervision; **Mordmüller B**: Funding Acquisition, Writing – Review & Editing; **Bousema T**: Funding Acquisition, Supervision, Validation, Writing – Review & Editing; **Graumans W**: Investigation, Methodology, Validation, Writing – Original Draft Preparation

Competing interests: No competing interests were disclosed.

Grant information: This specific work was partly supported by a European Research Council (ERC) Consolidator Grant to Teun Bousema [ERC-CoG 864180; QUANTUM]; and partly by the UK National Institute for Health Research (NIHR) Oxford Biomedical Research Centre (BRC). The views expressed are those of the authors and not necessarily those of the UK NIHR or the Department of Health and Social Care. The RaViCHMI1 clinical trial was funded by the European Union's Horizon Europe program under grant agreement No. 101080744 (OptiViVax). It also received co-funding from UK Research and Innovation (UKRI) under the UK government's Horizon Europe funding guarantee (No.: 10077974) and Swiss Government's State Secretariat for Education, Research, and Innovation (No.: 23.00182).
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Copyright: © 2026 Geraedts TJM *et al*. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Geraedts TJM, Chali W, van Gemert GJ *et al.* **Optimizing *Plasmodium vivax* gametocyte enrichment to enhance transmission to *Anopheles* mosquitoes [version 1; peer review: 2 approved with reservations]** Open Research Europe 2026, **6**:252 https://doi.org/10.12688/openreseurope.23635.1

First published: 22 Jul 2026, **6**:252 https://doi.org/10.12688/openreseurope.23635.1

Background

Malaria remains a major public health problem with an estimated 282 million malaria cases in 2024.[1] Most of the cases were caused by *Plasmodium falciparum*, the deadliest parasite species that is dominant in sub-Saharan Africa. *P. vivax* is the second leading cause of malaria worldwide and is mostly associated with malaria-related disease burden in South and Southeast Asia, and South America.[2] However, *P. vivax* also contributes substantially to malaria cases on the African continent, where it was long considered absent due to the high prevalence of Duffy negativity.[3] This is supported by a recent study from Ethiopia that demonstrates *P. vivax* can infect Duffy-negative individuals.[4] Recognizing this substantial disease burden, the World Health Organization has prioritized *P. vivax* research in its Malaria Vaccine Technology Roadmap.[5]

P. vivax biology differs markedly from *P. falciparum* and poses unique challenges.[6] One of its most distinctive features is the ability to form hypnozoites, dormant liver stage parasites that can reactivate weeks to months after the initial infection and are responsible for most recurrent episodes.[7] The study of *P. vivax* and their hypnozoite formation would benefit from a reliable source of viable sporozoites. However, the reproducible establishment of long-term continuous *in vitro* culture of *P. vivax* has so far proven to be an unattainable goal.[8] This is largely due to *P. vivax* exclusively invading immature red blood cells, so-called reticulocytes. As a result, studying sporozoites and hypnozoites continues to be dependent on mosquitoes infected by natural gametocyte carriers, typically involving patients at remote field sites where experimental conditions can be challenging. Although natural infections can reach high parasite densities, and *P. vivax* gametocytes appear early in infection, mosquito infection success remains variable and unpredictable.[9] These challenges pose a relevant bottleneck for research on pre-erythrocytic vaccines as well as for studies on hypnozoites. A method to achieve higher and more reproducible mosquito infection would be highly valuable for downstream applications.

Two gametocyte enrichment approaches have proven to be effective for *P. falciparum*: magnetic-activated cell sorting (MACS), exploiting the paramagnetism of mature gametocytes,[10–12] and Percoll density gradient centrifugation (DGC), separating infected erythrocytes by density.[13] While both methods have been repeatedly used for *P. falciparum*, information on their suitability for *P. vivax* is sparser.[14–16] Here, we utilized gametocyte-positive blood samples from a *P. vivax* PvW1 controlled human malaria infection (CHMI) to systematically compare both techniques and establish a protocol that achieves the highest proportion of infected mosquitoes and the highest infection burden (oocyst density) in these mosquitoes. We also confirm completion of sporogonic development in mosquitoes by detecting sporozoites in mosquito salivary glands.

Methods

Plasmodium falciparum and *Plasmodium vivax* gametocytes

Concentration methods were first tested using *P. falciparum* gametocytes (NF54) that were cultured in a tipper system,[17] as previously described.[18] Mosquito feeding experiments with *P. falciparum* were performed on day 14 post seeding, by spiking pelleted cultured gametocytes at a concentration of 0.75 million/mL into 10 mL fresh heparinized blood (ref 367526, BD). *P. vivax* gametocytes were the primary focus of this study and obtained during the RaViCHMI1 clinical trial (ID NL-OMON57011) that will be published separately. Briefly, study participants were inoculated intravenously with the blood-stage *P. vivax* W1 challenge inoculum (PvW1), provided by the University of Oxford.[19] PvW1 is a clonal parasite line from Thailand that has been cryopreserved in 2021.[20] *P. vivax* mosquito feeding experiments were conducted using blood samples from eight participants with a positive thick blood smear at a parasitemia between 2–38 parasites/µL between day 18 to 24 post infection (ID NL-OMON57011). Heparinized blood was obtained from study participants by venipuncture and immediately transferred to a water bath set at 37 °C until sample processing. All additional blood products required were supplied by the national blood bank (Sanquin Nijmegen, The Netherlands).

Mosquito husbandry and *Plasmodium* infection

Anopheles stephensi mosquitoes of the Sind-Kasur strain were reared at 30 +/− 1 °C and ~ 80% relative humidity, in a room with a reversed light period (12 h shifts).[21] *P. vivax* infected mosquitoes received an extra uninfected blood-meal on day 6 post-infection to harmonize oocyst and sporozoite development[22] and were housed at 26 +/− 1 °C with ~80% relative humidity. For all membrane feeding assays *An. stephensi* mosquitoes were allowed to feed for 10 min in the dark on blood in water-jacketed membrane feeders. For the membrane feeding assay with un-enriched gametocytes, about 100 female mosquitoes between 1–3 days old were collected in cages and fed using midi glass feeders (approximately 1.2 mL).[23] For gametocyte enriched feeds, about 50 female mosquitoes (1–3 days old) were collected in cups and fed using mini glass feeders that contained approximately ~300 µL material.[23] Unfed and partially fed mosquitoes were removed. Fully fed mosquitoes were maintained on 5% glucose (D+ Glucose Monohydrate, ref 49159, Sigma) and dissected 5 to 8 days post feeding to count the number of developed oocysts on midguts by microscopy after 1% mercurochrome staining.

Membrane feeding assays

All steps until mosquito feeding, including centrifugation, were performed at 37 °C to prevent early activation of gametocytes and thereby a potential loss of infectivity. Blood meals were prepared with lithium heparinized blood (ref 367526, BD) for *P. falciparum* cultured gametocytes, or with blood obtained by venipuncture (again in lithium heparin tubes) from participants enrolled in a *P. vivax* CHMI. The non-enriched blood meal (control) for *P. falciparum* was prepared as previously described.[24] For *P. vivax*-infected blood, plasma for the non-enriched feed was replaced with malaria-naive serum (group AB; Sanquin blood bank, The Netherlands).

Prior to gametocyte-enrichment, both *P. falciparum* cultured gametocytes and *P. vivax* gametocyte-infected in whole blood were leukodepleted by Plasmodipur (Europroxima) filtration. After flushing the filter with 2 mL pre-warmed RPMI medium, 10 mL blood material was gently filtered into a 15 mL tube. Tubes were spun for 5 min at 760 *g* after which plasma was removed without disturbing the top layer of erythrocytes. Blood was then brought to a hematocrit of 50% by resuspension with pre-warmed RPMI medium.[18]

The MACS procedure was performed as previously described,[12] with minor modifications. Briefly, columns were attached to the QuadroMACS™ separator (Miltenyi Biotech) in a 37 °C incubator, a hypodermic needle (25G) was attached, and the column was equilibrated with 1 mL pre-warmed RPMI medium. Subsequently, 3 mL of blood diluted in RPMI (50% hematocrit) was loaded onto the column; this was repeated on the same column until a total volume of 10 mL blood was processed. Columns were subsequently washed two times with 1 mL RPMI medium to remove unbound erythrocytes (referred to as wash 1 and wash 2). Columns were then detached from the magnet and placed in 15 mL tubes. Twice 1 mL RPMI medium was added to collect the bound fraction; the plunger that was provided with the column was used after the second 1 mL medium addition to expel all the retained liquid. Collection tubes were spun at 760 *g* for 1 min. The supernatant was carefully removed and approximately 1 mL was left on top of the pellet.

Percoll density gradient centrifugation was performed as previously described,[13] with minor modifications. A 90% isotonic Percoll (SIP) solution (Cytiva, 17089101) was prepared with PBS (10x) on the day of use. The SIP solution was subsequently used to prepare 65% and 70% Percoll working solutions (v/v) in PBS (1x). 6 mL aliquots were prepared in 15 mL tubes and pre-warmed at 37 °C. The 10 mL blood sample was divided over 3 Percoll tubes as follows: a maximum of 3.3 mL blood (50% hematocrit) was gently layered via the wall of the tube onto the Percoll, using a 3 mL syringe with blunt needle. Tubes were spun at 760 *g* or 1500 *g* for 15 min at 37 °C without break. A 3 mL syringe with blunt needle was used to collect and transfer the layer on top of the gradient to a new 15 mL tube. Samples were topped up to 12 mL with pre-warmed RPMI and spun at 760 *g* for 5 min at 37 °C. The supernatant was carefully removed without disturbing the pellet, and the washing procedure was repeated two times. After the final wash, 0.5 mL of supernatant was left on top of the pellet. Subsequently, pellets from three tubes (from one study participant) were resuspended and combined.

The resuspended pellet obtained by MACS or DGC was transferred to a 1.5 mL Eppendorf tube in a heating block set at 37 °C. 180 µL of pelleted erythrocytes from a universal donor (for *P. falciparum*) or from the same clinical trial participant were added. Tubes were spun in a benchtop centrifuge for 20 s at 10,000 rpm. The supernatant was very carefully removed, and the pellet was resuspended in 150 µL serum (group AB). The complete protocols can be found at protocol.io (Extended data).

Plasmodium vivax sporozoite quantification by microscopy

A subset of mosquitoes was dissected between day 11 and 20 post-blood feeding to collect the salivary glands, collecting 2 glands with 3 lobes each, into a glass pestle grinder containing 1 mL PBS (1x). After tissue homogenization, 10 µL from the suspension was loaded into a Bürker-Türk counting chamber that was left for 20 min at RT to allow sporozoites to settle. Sporozoites were counted using a phase contrast light microscope with a final magnification of 400x.

Plasmodium vivax quantification by q (RT)PCR

To quantify *P. vivax* parasites in material collected during MACS processing, 100 µL sample was collected in a 1.5 mL Eppendorf tube and mixed with 400 µL of RNA protect Cell Reagent (Qiagen). Samples were stored at −20 °C and extracted within 10 weeks post-collection. Total nucleic acid (NA) was extracted with the automated MagNAPure 96 instrument (Roche) using the MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche, Basel, Switzerland) and eluted in 50 µL. The number of *P. vivax* parasites was quantified by 18S rRNA quantitative PCR (qPCR) as described previously,[25] using the forward- 5′-GCTTTGTAATTGGAATGATGGGAAT-3′ and reverse primer 5′- ATGCGCA-CAAAGTCGATACGAAG −3′. A probe was used for amplicon detection 6FAM-AGCAACGCTTCTAGCTTA-MGBNFQ. *P. vivax* gametocytes were quantified by qRT-PCR, amplifying pvs25 as described previously,[25] using the forward- 5′- ACACTTGTGTGCTTGATGTATGTC -3′ and reverse primer 5′- ACTTTGCCAATAGCACATGAG-CAA −3′ set. A probe was used for amplicon detection 6FAM-TGCATTGTTGAGTACCTCTCGGAA-BHQ1. The

number of parasite copies was calculated with a standard curve, obtained using 10-fold dilutions of plasmids as previously described by Tadesse et al.[26] The MACS-column binding efficiency (%) was calculated by dividing copies in the bound fraction against the the total number of copies in the sample (total copies in bound + flow-through + wash 1 and 2 fractions).

Statistical analysis

Statistical analyses were performed using R (version 4.3.2). For oocyst density, values were analysed on the log10 scale. We fitted a linear model with condition (direct vs each enrichment method) as a categorical predictor and tested each enrichment method against the direct control using planned pairwise contrasts, reporting estimated mean differences with 95% confidence intervals and two-sided p-values. For ease of interpretation, these estimated differences on the log10 scale were back-transformed to mean fold changes (ratios) by exponentiation, yielding the enrichment-to-control oocyst density ratio. We analyzed the percentage infected mosquitoes using a linear model with condition as predictor, again testing each enrichment method versus the direct non-enriched control. Data are presented as violin plots with 95% confidence intervals. Correlations in proportion of infected mosquitoes and oocyst density between control and enrichment methods were visualized as scatter plots with dashed lines indicating a 1:1 reference. The relationship between oocyst prevalence and oocyst density was assessed using a logistic regression model, and 95% confidence intervals were calculated for the model fit. The significance level was set at a two-tailed $p < 0.05$.

Results

Optimizing *P. falciparum* gametocyte enrichment to enhance mosquito infection

The enrichment protocols were first examined for *P. falciparum* gametocytes. Methods tested were MACS with LS columns and two DGC gradients with two centrifugation speeds: Percoll 65%[14] (P65) and 70%[15] (P70) spun at a centrifugation speed of 760 *g*[11] or 1500 *g*[14] (Figure 1). All gametocyte enrichment methods increased the oocyst density and the proportion of infected mosquitoes (Figure 2A, B) relative to the non-enriched sample (control). Mean oocyst densities were as follows: control, 3.0 (range 0.6–6.2); MACS, 11.0 (range 4.9–21.2); DGC65-760 *g* 16.1 (7.9–29.4);

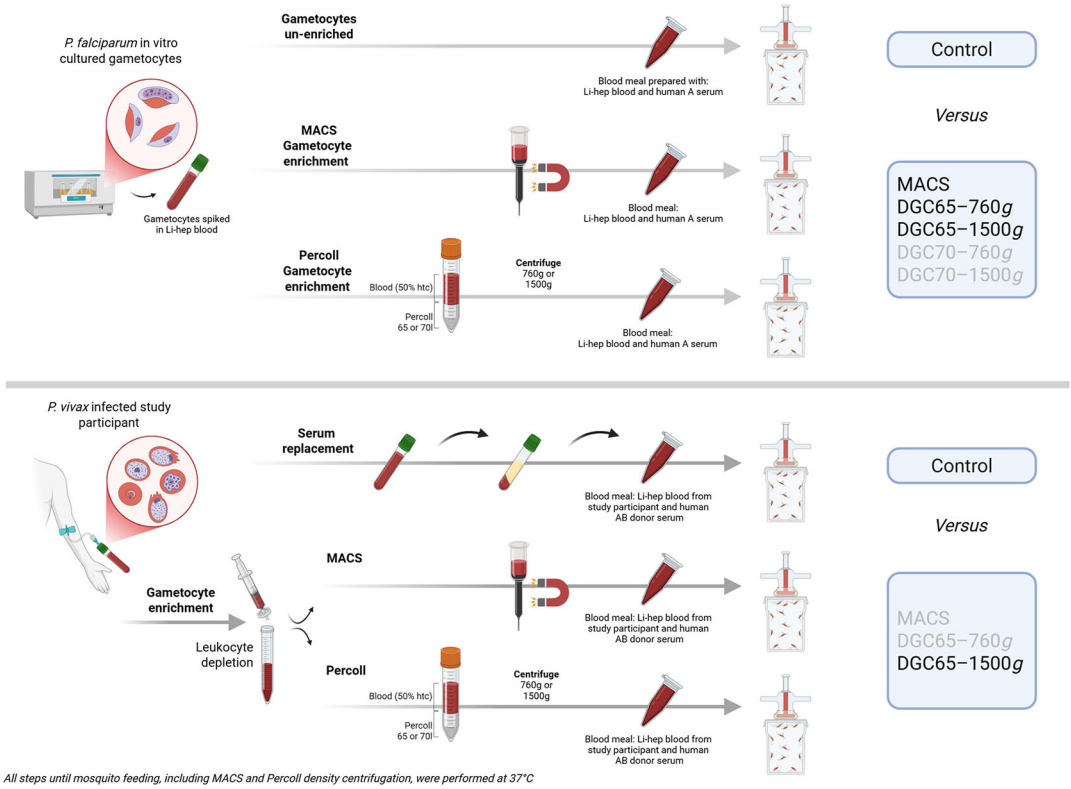

Figure 1. Membrane feeding assays performed with *P. falciparum* and *P. vivax* gametocytes. *Plasmodium falciparum* (*in vitro* cultured) and *P. vivax* (*ex vivo*) gametocytes were MACS and Percoll enriched, and fed to *Anopheles stephensi* mosquitoes to compare the proportion of infected mosquitoes and oocyst density against non-enriched blood (control). The boxes on the right of the figure indicate the tested control or enrichment conditions; enrichment conditions presented in grey were tested but found to be inferior to those presented in black.

DGC65–1500 *g*, 10.0 (6.3–16.1); DGC70–760 *g*, 17.4 (11.2–23.6); DGC70–1500 *g*, 21.2 (18.6–23.8) (**Extended data**). Mean infection rates were control, 66% (76/116); MACS, 88% (105/119); DGC65-760 *g*, 98% (55/56); DGC65-1500 *g*, 85% (51/60); DGC70-760 *g*, 90% (36/40); DGC70-1500 *g*, 97% (36/37) (**Extended data**). DGC70 enrichment resulted in the highest oocyst densities (Figure 2B) compared with control. DGC70-760 *g* and 1500 *g* increased oocyst density 7.5-fold (95% CI 2.0–29.1; p = 0.007) and 9.8-fold (95% CI 2.5–37.6; p = 0.004), respectively. The proportion of mosquitoes that became infected similarly increased with absolute increases of 24.8% (95% CI −1.8 to 51.4; p = 0.06) and 31.9% (95% CI 5.2–58.5; p = 0.02) for DGC-760 *g* and 1500 *g*, respectively (Figure 2A). DGC65–760 *g* achieved a 6.3-fold increase in oocyst density compared with control (95% CI 1.9–21.1; p = 0.006) (Figure 2B). This was accompanied by an absolute increase in the proportion of infected mosquitoes of 33.1% (95% CI 9.3–57.0; p = 0.01) (Figure 2A). In comparison, DGC65–1500 *g* and MACS resulted in smaller increases in oocyst density of 4.3-fold (95% CI 1.3–14.2; p = 0.02) and 4.4-fold (95% CI 1.3–14.8; p = 0.02), respectively. Their effects on the proportion of infected mosquitoes were comparable to those observed with DGC70–760 *g*, with absolute increases of 19.8% (95% CI 4.0–43.6; p = 0.09) for DGC65–1500 *g* and 23.1% (95% CI −0.7 to 46.9; p = 0.06) for MACS.

Although enrichment of *P. falciparum* gametocytes with DGC70 was achieved in two experiments, blood occasionally failed to penetrate the gradient after centrifugation (data not shown). Because this was observed repeatedly, independent of centrifugation speed, this condition was not considered in further evaluations. Both DGC65 and MACS were further assessed for their applicability in enriching *P. vivax* gametocytes.

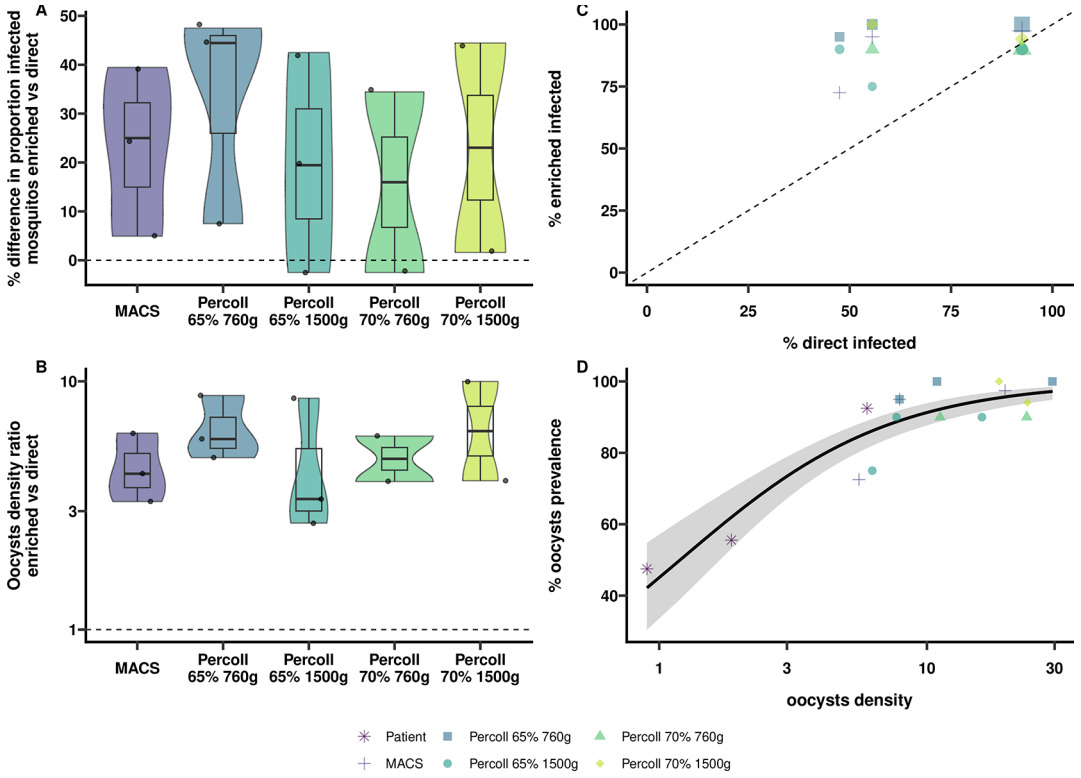

Figure 2. Comparing enrichment methods for *P. falciparum* cultured gametocytes. Blood from healthy malaria-naive blood donors was supplemented with mature cultured *P. falciparum* gametocytes. Non-enriched blood (control) was compared to gametocyte enriched blood (volume 2.5 mL) in the membrane feeding assay, using *Anopheles stephensi* mosquitoes and mini-feeders (300 µL). (**A**) Violin plots with 95% CI comparing the difference in proportion of infected mosquitoes (%) between control and gametocyte enriched blood feeding, using MACS and Percoll density gradient centrifugation (at two concentrations and spinning speeds). The dashed horizontal line shows a difference of 0 (i.e. equality). (**B**) Violin plots with 95% CI comparing mosquito oocyst density ratio of control feeding versus feeding after gametocyte enrichment method. Dashed horizontal line shows a ratio of 1 (i.e. equality). (**C**) Association of mosquito infection prevalence (%) between control and enriched-feeds, the size of the geometric figures depict the oocyst intensity. The dashed line shows a 1:1 association. (**D**) Trend for oocyst prevalence amongst positives versus oocyst density found by fitting a logistic regression model. The grey shaded area reflects the pointwise 95% confidence interval.

Comparing *P. vivax* gametocyte enrichment methods to enhance mosquito infection

Next, we evaluated *P. vivax* gametocyte enrichment by DGC65 (spun at 760 *g* or 1500 *g*) or MACS (LS columns); both were examined relative to the non-enriched control (Figure 1). Blood was drawn from eight study participants in a *P. vivax* blood-stage CHMI. Paired observations were collected for un-enriched versus enriched gametocytes with a total of 27 observations for DGC65-760 *g*, 28 for DGC65-1500 *g*, and 8 for MACS. For each condition, 11 mosquitoes were dissected to determine oocyst counts. DGC65-1500 g achieved the highest oocyst density and proportion of infected mosquitoes (Figure 3A, B). Compared with control, DGC65-1500 g increased the oocyst density 86-fold (95% CI 14–525; $p < 0.0001$) and increased the proportion of infected mosquitoes by 58.3% (95% CI 42.6–74.0; $p < 0.0001$). More modest effects were observed with DGC65-760 *g* that increased oocyst density 11-fold (95% CI 2.9–42.6; $p < 0.001$) and the proportion of infected mosquitoes by 28.0% (95% CI 12.1–43.9; $p < 0.001$) (Figure 3A, B). In contrast to all DGC conditions, MACS enrichment did not improve *P. vivax* mosquito infection, showing no increase in oocyst density (density ratio 0.68; 95% CI 0.09–5.28; $p = 0.71$) or in the proportion of infected mosquitoes (−5.4%; 95% CI −29.6 to 18.9; $p = 0.66$) compared to control (Figure 3A, B).

Gametocyte enrichment using DGC65 improved transmission significantly (Figure 3C). This was most apparent when mosquitoes were infected with blood containing low parasite densities, with 567 parasites/mL being the lowest parasitemia at which we observed transmission. DGC at a speed of 1500 *g* was superior and resulted in a higher oocyst intensity compared to 760 *g* (Figure 3D).

A subset of mosquitoes that fed on DGC65-1500 *g* gametocyte enriched blood were salivary gland dissected between day 11 and 20 post-blood feeding for quantification of sporozoites. Mean sporozoite load increased approximately proportionally with oocyst density in a weighted log–log regression model, adjusting for days post-infection relative to day

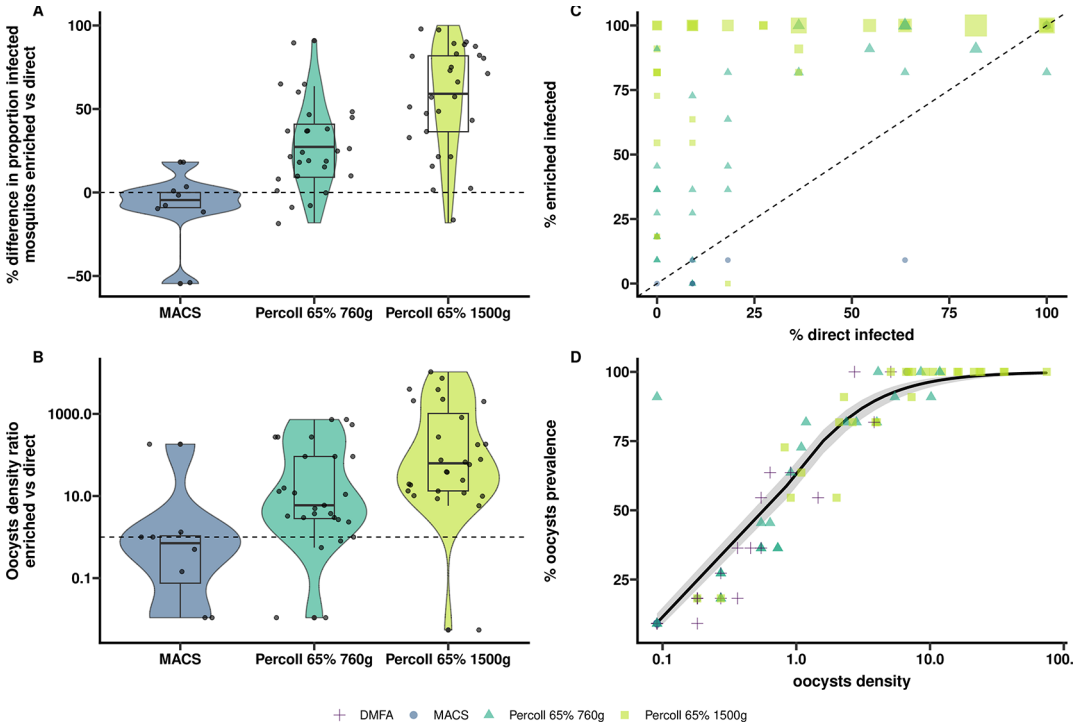

Figure 3. Comparing enrichment methods for *P. vivax* gametocytes. Gametocyte infected blood drawn from *P. vivax* CHMI study participants was fed non-enriched (control) and enriched (start volume 10 mL) to *Anopheles stephensi* mosquitoes in membrane feeders (300 µL). (**A**) Violin plots with 95% CI comparing the difference in proportion (%) of infected mosquitoes between non-enriched and gametocyte enriched blood feeding utilizing MACS and Percoll density gradient centrifugation (65%) using two different spinning speeds. The dashed horizontal line shows a difference of 0 (i.e. equality). (**B**) Violin plots with 95% CI comparing mosquito oocyst density ratio of control versus gametocyte enriched blood. Dashed horizontal line shows a ratio of 1 (i.e. equality). (**C**) Association of mosquito infection prevalence (%) between non-enriched and enriched-feeds, the size of the geometric figures depict the oocyst intensity. Dashed line shows a 1:1 association. (**D**) Trend for oocyst prevalence amongst positives versus oocyst density found by fitting a logistic regression model. The grey shaded area represents the pointwise 95% confidence interval.

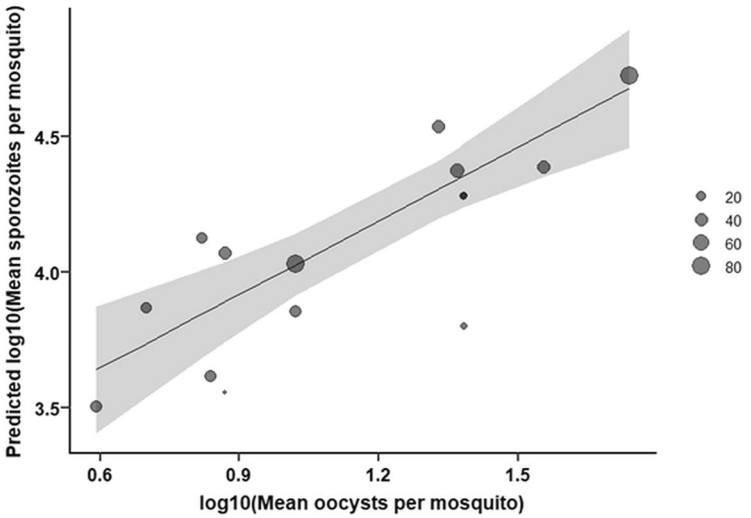

Figure 4. Model-predicted relationship between oocyst density and sporozoite load. Plot shows the predicted log10-transformed mean sporozoite load based on log10-transformed oocyst density from a weighted log-log regression model, adjusting for days post-infection relative to day 14. The shaded area represents the 95% confidence interval around the predicted relationship; point size reflects the number of dissected mosquitoes that ranged from 7–77.

14 (β = 0.91, 95% CI [0.51, 1.30], p = 0.0003). The model-predicted relationship between oocyst density and sporozoite load shows that oocyst burden and sporozoite load were strongly associated (Figure 4).

Effect of leukocyte depletion on *P. vivax* mosquito infection

A subset of 7 Percoll-enriched gametocyte samples was processed with and without filtration to explore whether leukocyte depletion by Plasmodipur filtration is an essential component to increase *P. vivax* transmission. For the proportion of infected mosquitoes, absolute differences between filtered and unfiltered conditions were small and not statistically significant: 9.09% (95% CI: −47.30 to 65.49; p > 0.99) for 1500 *g*, and − 0.00% (95% CI: −89.17 to 89.17; p > 0.99) for 760 *g*. Differences in oocyst intensity were similarly negligible: 0.29 (95% CI: −1.00 to 1.57; p = 0.96) for 1500 *g*, and 0.16 (95% CI: −1.87 to 2.19; p = 0.96) for 760 *g*.

Quantifying binding of *P. vivax* gametocytes to MACS LS and LD columns

As detailed above, *P. vivax* gametocyte enrichment by MACS did not result in increased mosquito infection prevalence and intensity compared to the control conditions. To explore whether this was due to inefficient column binding, we molecularly quantified enriched parasites. Blood from two study participants was processed in parallel on LS and LD columns and the following fractions were collected: starting, flow-through, wash 1, wash 2, and bound (as described elsewhere[11]). Parasites were quantified by qPCR targeting 18S (total parasites) and Pv25 (gametocytes) transcripts.[24] The proportion of *P. vivax* gametocytes in the bound fraction was 0 and 0.1% when using LS columns and 17.7 and 33.6% when using LD columns (**Extended data**).

Discussion

This study aimed to determine the best method to enrich *P. vivax* gametocytes out of whole blood samples to enhance mosquito infection. We demonstrated that Percoll density gradient centrifugation at a concentration of 65%, when centrifuged at a speed of 1500 *g*, results in the highest mosquito infection and oocyst density. While MACS can successfully enhance mosquito infectivity for *P. falciparum* gametocytes, we observed no increase when the method was applied on *P. vivax* gametocytes which was plausibly due to inefficient column-binding of gametocytes. Our optimized *P. vivax* enrichment protocol allows for reproducible mosquito infections even at low-density gametocyte infections.

Two previous studies evaluated MACS and Percoll enrichment for *P. vivax* gametocytes.[15,16] A study of Vera *et al*. used naturally infected malaria patients, with parasitemia ranging from 297.5 to 30,600 parasites/μL (of which 13.3% ± 16.7% gametocytes), to develop a method to purify and concentrate viable gametocytes. This study compared enrichment using DGC45, DGC60 and MACS, with DGC60 resulting in a significantly higher percentage of gametocytes. However, gametocyte infectivity was only accessed for DGC45, with a limited number of mosquito membrane feedings (n = 8) and without parallel control feeds of unenriched material. We can therefore not directly compare our findings to their work. A

study of Ramos *et al*. used *P. vivax* gametocytes from naturally infected participants, with parasitemia ranging from 501–10,000 parasites/μL, to evaluate viability of enriched gametocytes after short-term culture. Gametocytes were enriched using DGC70 and MACS and fed to mosquitoes at different time intervals post enrichment. Their independent assays allowed them to study gametocyte viability over time for each condition, but comparison between enrichment methods or to a control was not possible. Both studies reported gametocytemia as the proportion of gametocytes relative to total parasites (gametocytes/total parasites). This measure reflects purity (i.e. the depletion of asexual parasites) rather than yield (the absolute number of gametocytes recovered). Consequently, enrichment may appear successful even if substantial numbers of gametocytes are lost during processing— this would only become evident when gametocyte numbers are quantified, or infectivity is assessed in mosquito feeding assays as we did in the current study. We therefore consider our study of value to formally compare the effect of different methods of gametocyte enrichment on transmission.

To our surprise, MACS was successful in enhancing mosquito infection for *P. falciparu*m gametocytes, which was in line with previous studies,[11,12,27–29] but not for *P. vivax.* To explore why MACS LS columns did not result in a measurable increase in mosquito infection rates, we determined the fraction of gametocytes in the bound and flow-through fractions for both LS- and LD columns in a small set of experiments. In these experiments MACS LS columns failed to retain *P. vivax* gametocytes effectively (**Extended data**). An increase in enrichment was observed when using MACS LD columns, which are specifically designed to capture weaker magnetic cells. One possible explanation for this observation is that *P. vivax* gametocytes have a different hemozoin content or intracellular distribution, resulting in reduced paramagnetic properties. Even though no direct comparative data on hemozoin levels between *P. vivax* and *P. falciparum* is available, the distribution of hemozoin in gametocytes is morphologically different: the hemozoin in the cresent shaped *P. falciparum* gametocyte is more condensed compared to *P. vivax*, which is oval shaped with a more dispersed pattern. This may result in different paramagnetic properties and could explain the difference in MACS enrichment between the two parasite species. Dedicated studies using methods such as quantitative electron microscopy or magneto-optical assays to study absolute hemozoin concentration in both *P. vivax* and *P. falciparum* gametocytes could help support this hypothesis further. Of note, even the better performing LD columns did not achieve binding efficiencies in the range of those observed for *P. falciparum.*[12]

Beyond poor retention, impaired gametocyte quality caused by the MACS process may have further contributed to the poor transmission results we observed. The MACS magnetic field could induce oxidative or mechanical stress. Such stress-related damage may continue to accumulate even after removal from the magnetic field, ultimately impairing infectivity even if gametocytes are more efficiently enriched by LD columns. This damage may be more pronounced in *P. vivax* gametocytes because they preferentially invade reticulocytes, which are considered fragile.[8] This hypothesis is supported by data from the Ramos *et al.* study, where they show a more rapid decline in infectivity of MACS-enriched gametocytes compared to Percoll — infectivity dropped after 6 hours of culture for MACS versus up to 12 hours for Percoll.[15] Because we did not perform mosquito feeding assays following enrichment with MACS LD columns, the effect of the MACS magnetic field on *P. vivax* gametocytes remains uncertain.

This study has some limitations. First, we did not quantify gametocyte densities prior to enrichment or mosquito feeding. We are therefore unable to quantify and compare the gametocyte concentration efficacy of each enrichment method. Second, a subset of experiments was performed for protocol optimization and did not have appropriate power to detect statistically significant differences. Of particular interest were the experiments omitting leukodepletion. Even though we were unable to show statistical significance, we have shown that, in this small sample size, omitting the Plasmodipur filter was not detrimental to mosquito infectivity. A protocol without the need for leukodepletion would lower costs significantly (Plasmodipur filter costs ~25 €) and reduce handling time, making the methodology more accessible. For large scale experiments, the reduction in handling time may become particularly important since all procedures must take place at 37 °C.

In conclusion, we established the optimal methodology to reliably generate highly infected *P. vivax* mosquitoes through optimized gametocyte enrichment, even at low parasitemia. The resulting infected mosquitoes also completed sporozoite development, demonstrating the suitability of the protocol for downstream applications such as evaluating pre-erythrocytic interventions and studies on hypnozoite biology.

Ethics and consent

Written informed consent was obtained from CHMI volunteers who participated in the RaViCHMI1 clinical trial (ID NL-OMON57011), this study was approved by the Central Committee for Research Involving Human Subjects in The Netherlands (CCMO; NL-005553).

Data availability

Underlying data

Radboud Data Repository: *P. vivax* gametocyte enrichment. https://doi.org/10.34973/yhh7-0726.[30]

This project contains the following underlying data:

- Oocyst_data (raw oocyst counts per mosquito)
- Sporozoite_data (mean sporozoite counts per mosquito pool)

Extended data

Radboud Data Repository: P. vivax gametocyte enrichment. https://doi.org/10.34973/yhh7-0726.[30]

This project contains the following extended data:

- Results of cultured *P. falciparum* gametocytes prior (unenriched control) or after enrichment methods
- The proportion of *P. vivax* parasites (18S) and gametocytes (Pv25) during MACS sample processing

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

The enrichment protocols can be found at protocol.io: https://dx.doi.org/10.17504/protocols.io.ewov1rbkklr2/v1.

Acknowledgements

The authors are grateful to Jolanda Klaassen, Laura Pelser-Posthumus and Astrid Pouwelsen for their role in mosquito husbandry and extrapolating data from the *P. vivax* infected mosquitoes. We thank Weronika Machnik and Eyob Addise Workneh for technical assistance during sample processing; the BIO-006 study team (University of Oxford) and the OptiViVax Project Management team for assistance; and all the study participants of the RaViCHMI1 trial.

References

1. Organization, W.H: **World malaria report.** 2024.
2. Battle KE, Baird JK: **The global burden of Plasmodium vivax malaria is obscure and insidious.** *PLoS Med.* 2021; **18**(10): e1003799.
PubMed Abstract | Publisher Full Text | Free Full Text
3. Twohig KA, Pfeffer DA, Baird JK, *et al.*: **Growing evidence of Plasmodium vivax across malaria-endemic Africa.** *PLoS Negl. Trop. Dis.* 2019; **13**(1): e0007140.
PubMed Abstract | Publisher Full Text | Free Full Text
4. Bradley L, Yewhalaw D, Hemming-Schroeder E, *et al.*: **Epidemiology of Plasmodium vivax in Duffy negatives and Duffy positives from community and health centre collections in Ethiopia.** *Malar. J.* 2024; **23**(1): 76.
PubMed Abstract | Publisher Full Text | Free Full Text
5. (WHO), W.H.O: **Malaria Vaccine Technology Roadmap.** 2013.
Reference Source
6. Escalante AA, Cepeda AS, Pacheco MA: **Why Plasmodium vivax and Plasmodium falciparum are so different? A tale of two clades and their species diversities.** *Malar. J.* 2022; **21**(1): 139.
PubMed Abstract | Publisher Full Text | Free Full Text
7. Commons RJ, Simpson JA, Watson J, *et al.*: **Estimating the Proportion of Plasmodium vivax Recurrences Caused by Relapse: A Systematic Review and Meta-Analysis.** *Am J Trop Med Hyg.* 2020; **103**(3): 1094–1099.
PubMed Abstract | Publisher Full Text | Free Full Text
8. Bermudez M, *et al.*: **Plasmodium vivax in vitro continuous culture: the spoke in the wheel.** *Malar. J.* 2018; **17**(1): 301.
PubMed Abstract | Publisher Full Text | Free Full Text
9. Miura K, Swihart BJ, Fay MP, *et al.*: **Evaluation and modeling of direct membrane-feeding assay with Plasmodium vivax to support development of transmission blocking vaccines.** *Sci. Rep.* 2020; **10**(1): 12569.
PubMed Abstract | Publisher Full Text | Free Full Text
10. Karl S, Davis TM, St Pierre TG: **Short report: Quantification of Plasmodium falciparum gametocytes by magnetic fractionation.** *Am J Trop Med Hyg.* 2011; **84**(1): 158–160.
PubMed Abstract | Publisher Full Text | Free Full Text
11. Reuling IJ, Stone WJR, van de Vegte-Bolmer M, *et al.*: **Concentration of Plasmodium falciparum gametocytes in whole blood samples by magnetic cell sorting enhances parasite infection rates in mosquito feeding assays.** *Malar. J.* 2017; **16**(1): 315.
Publisher Full Text
12. Graumans W, Andolina C, Awandu SS, *et al.*: **Plasmodium falciparum Gametocyte Enrichment in Peripheral Blood Samples by Magnetic Fractionation: Gametocyte Yields and Possibilities to Reuse Columns.** *Am J Trop Med Hyg.* 2019; **100**(3): 572–577.
Publisher Full Text
13. Collins KA, Wang CYT, Adams M, *et al.*: **A controlled human malaria infection model enabling evaluation of transmission-blocking interventions.** *J. Clin. Invest.* 2018; **128**(4): 1551–1562.
PubMed Abstract | Publisher Full Text | Free Full Text
14. Collins KA, Wang CYT, Adams M, *et al.*: **A Plasmodium vivax experimental human infection model for evaluating efficacy of interventions.** *J. Clin. Invest.* 2020; **130**(6): 2920–2927.
PubMed Abstract | Publisher Full Text | Free Full Text
15. Ramos GQ, Baia-da-Silva DC, Lacerda MVG, *et al.*: **Viability and Infectivity of Plasmodium vivax Gametocytes in Short-Term Culture.** *Front. Cell. Infect. Microbiol.* 2021; **11**: 676276.
PubMed Abstract | Publisher Full Text | Free Full Text

16. Vera O, Brelas de Brito P, Albrecht L, *et al.*: **Purification Methodology for Viable and Infective Plasmodium vivax Gametocytes That Is Compatible with Transmission-Blocking Assays.** *Antimicrob. Agents Chemother.* 2015; **59**(10): 6638–6641.
PubMed Abstract | Publisher Full Text | Free Full Text

17. Ponnudurai T, Lensen AHW, Leeuwenberg ADEM, *et al.*: **Cultivation of fertile Plasmodium falciparum gametocytes in semi-automated systems. 1. Static cultures.** *Trans. R. Soc. Trop. Med. Hyg.* 1982; **76**(6): 812–818.
PubMed Abstract | Publisher Full Text

18. van de Vegte-Bolmer M, Graumans W, Stoter R, *et al.*: **A portfolio of geographically distinct laboratory-adapted Plasmodium falciparum clones with consistent infection rates in Anopheles mosquitoes.** *Malar. J.* 2021; **20**(1): 381.
PubMed Abstract | Publisher Full Text | Free Full Text

19. Minassian AM, Themistocleous Y, Silk SE, *et al.*: **Controlled human malaria infection with a clone of Plasmodium vivax with high-quality genome assembly.** *JCI Insight.* 2021; **6**(23).
Publisher Full Text

20. Salkeld J, Themistocleous Y, Barrett JR, *et al.*: **Repeat controlled human malaria infection of healthy UK adults with blood-stage Plasmodium falciparum: Safety and parasite growth dynamics.** *Front. Immunol.* 2022; **13**: 984323.
PubMed Abstract | Publisher Full Text | Free Full Text

21. Graumans W, *et al.*: **Comparative assessment of mosquito size and receptiveness to *P. falciparum* infection of two rearing procedures for *Anopheles* mosquitoes.** *bioRxiv.* 2023; 2023.03.09.531859.

22. Shaw WR, Holmdahl IE, Itoe MA, *et al.*: **Multiple blood feeding in mosquitoes shortens the Plasmodium falciparum incubation period and increases malaria transmission potential.** *PLoS Pathog.* 2020; **16**(12): e1009131.
PubMed Abstract | Publisher Full Text | Free Full Text

23. Graumans W, Heutink R, van Gemert GJ, *et al.*: **A mosquito feeding assay to examine Plasmodium transmission to mosquitoes using small blood volumes in 3D printed nano-feeders.** *Parasit. Vectors.* 2020; **13**(1): 401.
PubMed Abstract | Publisher Full Text | Free Full Text

24. Ponnudurai T, Lensen AHW, van Gemert GJA, *et al.*: **Infectivity of cultured Plasmodium falciparum gametocytes to mosquitoes.** *Parasitology.* 1989; **98**(Pt 2): 165–173.
Publisher Full Text

25. Tadesse FG, van den Hoogen L, Lanke K, *et al.*: **The shape of the iceberg: quantification of submicroscopic Plasmodium falciparum and Plasmodium vivax parasitaemia and gametocytaemia in five low endemic settings in Ethiopia.** *Malar. J.* 2017; **16**(1): 99.
PubMed Abstract | Publisher Full Text | Free Full Text

26. Tadesse FG, Slater HC, Chali W, *et al.*: **The Relative Contribution of Symptomatic and Asymptomatic Plasmodium vivax and Plasmodium falciparum Infections to the Infectious Reservoir in a Low-Endemic Setting in Ethiopia.** *Clin. Infect. Dis.* 2018; **66**(12): 1883–1891.
PubMed Abstract | Publisher Full Text

27. Karl S, David M, Moore L, *et al.*: **Enhanced detection of gametocytes by magnetic deposition microscopy predicts higher potential for Plasmodium falciparum transmission.** *Malar. J.* 2008; **7**: 66.
PubMed Abstract | Publisher Full Text | Free Full Text

28. Ribaut C, Berry A, Chevalley S, *et al.*: **Concentration and purification by magnetic separation of the erythrocytic stages of all human Plasmodium species.** *Malar. J.* 2008; **7**: 45.
PubMed Abstract | Publisher Full Text | Free Full Text

29. Ahn S: **Magnetic separation: a highly effective method for synchronization of cultured erythrocytic Plasmodium falciparum.** *Parasitol. Res.* 2008; **102**: 1195–1200.
PubMed Abstract | Publisher Full Text

30. Geraedts T, *et al.*: P. vivax gametocyte enrichment. [dataset]. Radboud University; 2026. Version 1.
Publisher Full Text

Open Peer Review

Current Peer Review Status: ? ?

Version 1

Reviewer Report 12 October 2026

https://doi.org/10.21956/openreseurope.25589.r81841

© **2026 Ubiaru P et al.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

?

Prince Chigozirim Ubiaru
[1] School of Biodiversity, One Health and Veterinary Medicine, University of Glasgow, Glasgow, UK
[2] Life Sciences, Medical School, University of Nottingham, Nottingham, UK
Omar Janha
University of Glasgow, Advanced Research Centre, Glasgow, Glasgow City, UK
Sabyasachi Pradhan
University of Glasgow College of Medical Veterinary and Life Sciences (Ringgold ID: 223860), Glasgow, Scotland, UK

This work is demonstrably a good starting point for allowing the study of transmission of *Plasmodium vivax* and offers the opportunity for oocyst and sporozoite studies. It can also be used to study early mosquito stages of *P. vivax* infection, and sporozoites can be used for vaccine development studies. However, we observed some important omissions that should be addressed to improve the manuscript and help the readers:

1. “An extra uninfected blood meal was done on day 6 post-infection to harmonize oocyst development”. However, mosquito dissection began on days 5 to 8 post-feeding to count developed oocysts. This implies the mosquitoes dissected on day 5 post-feeding missed the extra blood meal intended to harmonize oocyst infection, and the mosquitoes dissected on day 6 post-infection may not be able to digest the extra uninfected blood before midgut dissection. The authors need to justify this, as it could bias the mosquito infection data they presented, especially if one test experiment was dissected on days 5 and 6 post-feeding and the rest on days 7 and 8.

2. It appears that figure 1 was generated using figure creation tools or scientific diagram software but is not credited by referencing; authors should reference the tools used to generate this figure.

3. Also, in figure 1 legend, the authors stated: “the boxes on the right of the figure indicate the tested control or enrichment conditions; enrichment conditions presented in grey were found to be inferior to those presented in black”. This statement is inconsistent with the results the authors presented, especially in the *P. falciparum* data. See (Black; MACs = 88%, DGC65-1500g = 85% against Grey; DGC70-760g = 90%, DGC70-1500g = 97%).

4. The authors mentioned several times the use of gametocyte purification techniques: Percoll density gradient at different centrifugation speeds (1500g and 760g) as well as magnetic cell sorting (MACS) purification methods. However, the authors show no images of un-enriched (control) and enriched gametocytes. It would add significant value of information if the authors could add images to show how concentrated the enriched gametocytes are compared to the unenriched controls, since neither was quantified. This is particularly important since different concentrations of Percoll and spinning times were used, and the results indicated that gametocyte enrichment increases both infection prevalence and intensity.

5. The authors stated in the results section, “All gametocyte enrichment methods increased the oocyst density and the proportion of infected mosquitoes (Figure 2A, B) relative to the non-enriched sample (control).” In the extended data, it is shown that in experiment 2, the control prevalence was higher than some of the enrichment methods. Perhaps the authors should try to incorporate the control data points in their graph as well for a better understanding of the results in such cases. Also, there were no raw data (prevalence and intensity), like the *falciparum* data, provided for the *vivax* infection experiments. Having the control plotted alongside the test samples with p-value indications on the same graph (for both *falciparum* and *vivax* experiments) helps with better comparative visualisation of the results, since the authors suggests for both *falciparum* and *vivax* prevalence and intensity increase with enrichment.

6. The figure legend should state what the individual dots represent in figures 2A & B as well as 3A & B.

7. The authors stated that they “did not quantify gametocyte densities prior to enrichment or gametocyte feeding”, but they have not given any reason for not doing this. Gametocyte densities are important for establishing mosquito infection. If the number of gametocytes fed to the mosquitoes was not standardised between the different enrichment methods and the non-enriched control, then it would be difficult to compare between them, as they would be fed with different numbers of gametocytes, leading to different rates of infection. The enriched samples, like the 70% Percoll treated and longer centrifugation conditions, would be expected to have more gametocytes and fewer erythrocytes. Therefore, mosquitoes fed with these samples may have more gametocytes, which could increase the proportion of infected mosquitoes and oocyst density. Without knowing the gametocyte concentration in each feed, it is difficult to conclude whether the increase in the proportion of infected mosquitoes and oocyst density is an attribute of gametocyte quality or gametocyte quantity in the supplied feed. The authors should clarify this for both *P. falciparum* and *P. vivax* experimental sets and discuss how this may affect the interpretation of their results.

8. The authors report “Blood was drawn from eight study participants in a *P. vivax* blood-stage CHMI. Paired observations were collected for un-enriched versus enriched gametocytes with a total of 27 observations for DGC65-760 g, 28 for DGC65-1500 g, and 8 for MACS.” For each condition, 11 mosquitoes were dissected to determine oocyst counts.” The authors should explain why for MACS only 8 observations were made and not the same as in other experimental conditions.

Minor corrections

9. In the 3rd statement before the methods section, add (infection prevalence) after the highest proportion of infected mosquitoes.

10. Line 4 in methods: add mature after cultured in this statement: "by spiking pelleted cultured gametocytes at a concentration of 0.75 million/mL into 10 mL fresh heparinized blood"

Under mosquito husbandry and *Plasmodium* infection

11. Were the adult female mosquitoes starved for 12-24 hours before the infected blood meal? This is a lab-standard method used to stimulate female *Anopheles stephensi* to feed via membrane feeding. (see; PMID: 2263414 or https://doi.org/10.1038/s41467-017-00571-y)

Under membrane feeding assays

12. Protocol.io (Extended data). Correct to protocols.io.

References

1. Lopaticki S, Yang A, John A, Scott N, et al.: Protein O-fucosylation in Plasmodium falciparum ensures efficient infection of mosquito and vertebrate hosts. *Nature Communications*. 2017; **8** (1). Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Plasmodium falciparum transmission, Mosquito-Plasmodium interactions

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Reviewer Report 10 October 2026

https://doi.org/10.21956/openreseurope.25589.r81838

© 2026 Cassiano G. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

?

Gustavo Capatti Cassiano
Universidade NOVA De Lisboa (UNL), Lisboa, Portugal

This study evaluated methods for enriching *Plasmodium vivax* gametocytes to improve experimental infection of *Anopheles stephensi* mosquitoes. Using blood from eight participants in a controlled human malaria infection study, the authors compared Percoll density gradient centrifugation and magnetic cell sorting (MACS) with non-enriched samples. The use of paired non-enriched controls and the substantial overall number of mosquitoes examined are strengths of this study. However, some aspects of the experimental design, statistical analysis, and interpretation would benefit from clarification:

1. Although enriched and non-enriched conditions were paired within each sampling occasion, the 27–28 paired observations came from only eight participants. Could the authors clarify whether the analysis accounted for pairing within sampling occasions and repeated sampling of the same participant? A supplementary table listing each participant and sampling occasion, the starting parasite density, the infection outcome in the corresponding non-enriched feed, and the enrichment methods tested would help readers assess these comparisons and establish how many observations each participant contributed.

2. The Methods state that oocyst density was analysed on the log10 scale, but some feeds or mosquitoes may have had zero oocysts. How were zero counts handled before transformation?

3. The statement that the benefit of DGC65 enrichment was “most apparent” at low parasite densities is difficult to assess from Figure 3C, which does not appear to display starting parasite density. Could the authors present the paired infection outcomes in relation to starting parasite density and clarify whether this statement is supported by a formal analysis or represents a descriptive observation? Furthermore, the lowest density associated with transmission—567 parasites/mL, equivalent to 0.567 parasites/µL—is below the 2–38 parasites/µL range specified in the Methods. Please clarify which samples and measurement methods these values refer to, whether they were measured before or after processing, and how the two ranges relate.

4. Previous studies have reported enrichment of *P. vivax* gametocytes using MACS LS columns, including recovery of material capable of infecting mosquitoes (e.g., Ramos et al., 2021). How do the authors reconcile those findings with the very low pvs25 recovery measured in the LS-bound fraction here? It would be helpful to distinguish enrichment in gametocyte proportion and absolute recovery from the starting sample. Furthermore, the *P. falciparum* comparison provides a useful reference for method performance, but differences between cultured and participant-derived samples limit its ability to establish a species-specific explanation for the LS result.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Malaria parasite biology and antimalarial drug discovery.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.