Identification of the asymptomatic Plasmodium falciparum and Plasmodium vivax gametocyte reservoir under different transmission intensities

Cristian Koepfli\textsuperscript{1,2,3,\textdagger}, Wang Nguitragool\textsuperscript{4}, Anne Cristine Gomes de Almeida\textsuperscript{5,6}, Andrea Kuehn\textsuperscript{7}, Andreaa Waltmann\textsuperscript{1,2}, Eline Kattenberg\textsuperscript{1,8\textdagger\textdagger}, Maria Ome-Kaius\textsuperscript{1,2,6}, Patricia Raraa\textsuperscript{8}, Thomas Obadia\textsuperscript{9,10}, James Kazura\textsuperscript{11}, Wuelton Monteiro\textsuperscript{5,6}, Andrew W. Darcy\textsuperscript{12}, Lyndes Wini\textsuperscript{13,15,16,17}, Quique Bassat\textsuperscript{7,14,15,16,17}, Ingrid Felger\textsuperscript{18}, Jetsumon Sattabongkot\textsuperscript{19}, Leanne J. Robinson\textsuperscript{1,8\textdagger\textdagger}, Marcus Lacerda\textsuperscript{5}, Ivo Mueller\textsuperscript{1,2,10}

1 Population Health & Immunity Division, Walter & Eliza Hall Institute, Parkville, Australia, 2 Department of Medical Biology, University of Melbourne, Parkville, Australia, 3 University of Notre Dame, Eck Institute for Global Health, Department of Biological Sciences, Notre Dame, Indiana, United States of America, 4 Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, 5 Fundação de Medicina Tropical Dr. Heitor Vieira Dourado (FMT-HVD), Manaus, Brazil, 6 Universidade do Estado do Amazonas, Manaus, Brazil, 7 ISGlobal, Hospital Clinic—Universitat de Barcelona, Barcelona, Spain, 8 Papua New Guinea Institute of Medical Research, Madang, Papua New Guinea, 9 Hub de Bioinformática et Biostatistique, Département Biologie Computationnelle, Institut Pasteur, Paris, France, 10 Unité Malaria: parasites et Hôtes, Département Parasites et Insectes Vecteurs, Institut Pasteur, Paris, France, 11 Centre for Global Health & Diseases, Case Western Reserve University, Cleveland, Ohio, United States of America, 12 National Health Training and Research Institute, Ministry of Health, Honiara, Solomon Islands, 13 Vector Borne Diseases Program, Ministry of Health, Honiara, Solomon Islands, 14 Centro de Investigación en Saúde de Menhíca (CISM), Maputo, Mozambique, 15 ICREA, Barcelona, Spain, 16 Pediatric Infectious Diseases Unit, Pediatrics Department, Hospital Sant Joan de Déu (University of Barcelona), Barcelona, Spain, 17 Consorcio de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBERESP), Madrid, Spain, 18 Swiss Tropical and Public Health Institute, Basel, Switzerland, 19 Mahidol Vivax Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

\textsuperscript{\textdagger} Current address: Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium

\textsuperscript{\textdagger\textdagger} Current address: Burnet Institute, Melbourne, Australia

*ckoepfli@nd.edu

Abstract

**Background**

Understanding epidemiological variables affecting gametocyte carriage and density is essential to design interventions that most effectively reduce malaria human-to-mosquito transmission.

**Methodology/Principal findings**

Plasmodium falciparum and P. vivax parasites and gametocytes were quantified by qPCR and RT-qPCR assays using the same methodologies in 5 cross-sectional surveys involving 16,493 individuals in Brazil, Thailand, Papua New Guinea, and Solomon Islands. The proportion of infections with detectable gametocytes per survey ranged from 44–94% for P. falciparum and from 23–72% for P. vivax. Blood-stage parasite density was the most
important predictor of the probability to detect gametocytes. In moderate transmission settings (prevalence by qPCR>5%), parasite density decreased with age and the majority of gametocyte carriers were children. In low transmission settings (prevalence<5%), >65% of gametocyte carriers were adults. Per survey, 37–100% of all individuals positive for gametocytes by RT-qPCR were positive by light microscopy for asexual stages or gametocytes (overall: *P. falciparum* 178/348, *P. vivax* 235/398).

**Conclusions/Significance**

Interventions to reduce human-to-mosquito malaria transmission in moderate-high endemicity settings will have the greatest impact when children are targeted. In contrast, all age groups need to be included in control activities in low endemicity settings to achieve elimination. Detection of infections by light microscopy is a valuable tool to identify asymptomatic blood stage infections that likely contribute most to ongoing transmission at the time of sampling.

**Introduction**

A variety of malaria control interventions aim to reduce the transmission of parasites from the human to the mosquito host. Vector control tools such as bed nets and indoor residual spraying [1] lower the risk for infection in humans, and for onward transmission. Additional public health interventions primarily aimed at reducing human-to-mosquito transmission are currently being applied or developed, e.g. mass screening and treatment [2], mass drug administration [3], transmission blocking vaccines [4], and ivermectin administration [5].

Interventions that reduce human-to-mosquito transmission are most effective when they target individuals within a population who contribute most to transmission. Not all individuals with blood-stage parasitemia are equally infectious to mosquitoes. Only a small fraction of all parasites in the human host develop into sexual stages termed ‘gametocytes’; parasite
development in the mosquito relies on uptake and subsequent mating of male and female gametocytes [6].

Developing *P. falciparum* gametocytes are sequestered in extravascular sites such as bone marrow for 1–2 weeks [7, 8]. Gametocytes appear in the blood stream after the first wave of asexual parasites, and, in the case of symptomatic malaria cases, are detectable by microscopy often only 1–2 weeks after presentation with fever [9]. Mature gametocytes infective to mosquitoes circulate in peripheral blood for a period of a few days to up to three weeks [10–12]. Most commonly used antimalarials used to treat symptomatic cases do not clear sequestered or mature gametocytes. This is also the case for artemisinin combination therapy (ACT), the first-line drug in most *P. falciparum* endemic countries. As a result of the continued release of sequestered gametocytes after treatment, and their circulation for days to weeks, individuals can carry gametocytes for several weeks after treatment [12–14]. Primaquine is the only approved drug that clears *P. falciparum* gametocytes. Low-dose primaquine has been shown to reduce duration of gametocytemia after treatment [15, 16].

*P. vivax* gametocytes infective for mosquitoes appear within 2–3 days after blood-stage asexual parasitemia commences and might circulate for only 2–3 days [17, 18]. Gametocytes are present in the majority of clinical cases [19–21]. *P. vivax* gametocytes are sensitive to common drugs such as chloroquine or artemether-lumefantrine. In drug trials, up to 90% of patients were gametocyte-free one day after treatment, and virtually none carried gametocytes at day 7 [20–22].

In most malaria endemic regions the vast majority of infections are asymptomatic, i.e. not associated with fever. 50–80% of infections are not detectable by microscopic inspection of blood smears [23, 24]. Since gametocytes account for only a small proportion of all parasites in peripheral blood, they are more difficult to detect by light microscopy (LM) than asexual parasites. The development of molecular methods to detect gametocyte-specific RNA transcripts by nucleic acid sequence-based amplification (NASBA) or reverse-transcriptase quantitative PCR (RT-qPCR) has allowed for detection of submicroscopic gametocytemia [25, 26]. However, few studies have reported gametocyte carriage in asymptomatic individuals in non-African settings, especially with respect to *P. vivax* [27–33]. It is not known whether gametocyte densities differ across regions of different transmission intensities, and the distribution of gametocyte carriers among various demographic groups within a community is not well understood. Knowledge of these epidemiological variables is needed to target transmission-reducing interventions to those at highest risk of gametocyte carriage and to understand the long-term impact of these interventions on progress towards malaria elimination.

To advance our understanding of *P. falciparum* and *P. vivax* gametocyte carriage across a range of transmission settings, five cross-sectional surveys involving a total of 16,493 individuals were conducted in Brazil, Thailand, Papua New Guinea (PNG) and Solomon Islands [27–31]. The surveys included endemic areas where transmission was moderate to high or had recently decreased (PNG in 2010 and 2014, Solomon Islands) and regions where transmission was low, with *P. vivax* being the predominant parasite (Brazil, Thailand). Blood samples were collected from the general population irrespective of symptoms of malaria illness. Total blood-stage parasites and gametocytes were quantified by LM and sensitive qPCR and RT-qPCR assays using the same methodology across all studies, allowing for the first direct comparison across transmission intensities.

**Study sites and methods**

**Ethics statement**

The study was approved by the PNG Institute of Medical Research IRB (1116/1204), the PNG Medical Research Advisory Committee (MRAC numbers 11.21, 05.20, 12.06, 12.01), the
WEHI Human Research Ethics Committee (WEHI HREC, 12/09), the Case Western Reserve University Hospitals of Cleveland Medical Center (CWRU UHCMC, 05-11-11), the Solomon Islands National Health Research Ethics Committee (HRC12/022), the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand (EC approval number MUTM 2012-044-01), the Brazilian National Committee of Ethics (CONEP) (349.211/2013), and by the Committee of Ethics for Clinical Investigation from Barcelona Hospital Clinic (7306/2012). Prior to sample collection, the aims of the study were explained to all individuals and informed written consent was obtained from participants or, in case of minors, from their guardians.

Study sites and sample collection

Details on study sites are given in Table 1. Community sensitization took place 1–2 weeks prior to sample collection. Convenience sampling was applied to select households for the surveys. All members of the selected households were noted on a list, and individuals above 6 months of age were invited to participate. Sampling started in the morning and continued throughout the day. As children might be in school during this time and adults away for work, efforts were made to sample school-aged children and working adults after they returned home. The age and gender distributions of the study participants for each survey is given in Table 1, and is expected to be representative for the population. Overall, the distributions were similar across surveys, with the exception of a lower proportion of all sampled being small children in Brazil and Thailand.

The study sites experience little or moderate seasonality in transmission. In PNG, the rainy season is from December to April. Both surveys were conducted in May to July, i.e. after the rainy season. There is minimal seasonal variation in Solomon Islands; samples were collected in May to June. In Thailand, the peak transmission season is from April to July, and samples were collected in September and October. In Manaus, Brazil, highest incidence occurs from May to September. Half of the samples were collected in September to early January, and the other half in August to September.

Form each participant, 250 μL blood was collected by finger prick into 2 mL EDTA microtainers (BD). Hemoglobin levels were determined using the HemoCue handheld meter. For RNA extraction and gametocyte detection, 50 μL blood was transferred into tubes containing 250 μL of RNAprotect (Qiagen) in the field. Samples in RNAprotect were kept on ice packs in the field and transferred to -80˚C storage every evening and kept there until RNA extraction. The remaining 200 μL blood were kept in the EDTA microtainer, also kept on ice packs, and transferred to -20˚C storage until DNA extraction.

Table 1. Cross-sectional surveys included in this study.

| Survey Location                      | Year    | Individuals | Measures of recent malaria transmission                                                                 | Reference |
|--------------------------------------|---------|-------------|---------------------------------------------------------------------------------------------------------|-----------|
| PNG, Madang Province                 | 2010    | 2083        | Prevalence by PCR 41.7% *P. vivax* and 42.1% *P. falciparum* in 2006. After bed net roll out in 2007–2008, 4–5 fold decrease of clinical incidence in children, and 3-fold decrease of *P. vivax* molecular force of infection. | [27, 28] |
|                                      | 2014    | 2517        |                                                                                                         |           |
| Solomon Islands, Ngella, Central Islands Province | 2012    | 3501        | 10-fold decline in clinical incidence in last 20 years across Solomon Islands. In Tetere, Guadalcanal Province, prevalence by microscopy was 12.9% *P. falciparum* and 19.1% *P. vivax* in 2004–2005. | [30]     |
| Thailand, Kanchanburi and Ratchaburi Provinces | 2013    | 4309        | Prevalence by LM 0.1–5.2% *P. falciparum* and 0.2–5.9% *P. vivax* in 2000–2004.                           | [29]     |
| Brazil, Manaus                       | 2012–2013 | 4083       | Prevalence by PCR <1–2% *P. falciparum* and 8.5–15% *P. vivax* in different regions in the Amazon in 2002–2008. Since then 10-fold decrease in prevalence, virtually no *P. falciparum* left. | [31]     |

https://doi.org/10.1371/journal.pntd.0009672.t001
Molecular methods

DNA was extracted from 200 μL whole blood kept in EDTA microtainers and eluted in 200 μL elution buffer. Parasites were quantified by qPCR using the 18S rRNA gene as target [34]. The assay used detects one copy of the gene. 4 μL DNA was screened by qPCR, thus the limit of detection was 0.25 parasites/μL blood (i.e. 1 genome per 4 μL DNA). RNA was extracted from 50 μL whole blood kept in RNAProtect, and eluted in 50 μL elution buffer. 2 μL RNA was screened by RT-qPCR. Gametocytes were quantified by RT-qPCR of the female gamocyte-specific transcripts pf525 and pv525 [35].

Procedures for sample collection, qPCR, and RT-qPCR were standardized between all sites. Results of individual cross-sectional surveys have been published previously [28–31, 35]. For qPCR and RT-qPCR, standardized plasmids were distributed to all laboratories and run along samples for relative quantification and estimation of sensitivity. Sensitivity of all assays was 0.5–1 copies/μL DNA or RNA. For absolute measurements of copy numbers, a subset of samples from each laboratory was quantified by droplet digital PCR [36]. Due to different procedures of RNA sample collection in Solomon Islands, only positivity, but not pv525 and pf525 copy numbers, were included in the analysis. Expert microscopy was conducted in PNG, Solomon Islands, and for parts of the survey in Brazil. To determine multiplicity of infection (MOI), *P. falciparum* infections were genotyped by *msp2* [37], and *P. vivax* infections were genotyped by *msp1F3* and MS2 [38].

Data analysis

The following definitions are used: ‘proportion gametocyte-positive infections’ describes the number of gametocyte carriers divided by the number infected with asexual parasites and/or gametocytes; ‘population gametocyte prevalence’ is the prevalence of gametocytes among all individuals, infected and non-infected [39].

Multivariable regression models were used to predict factors associated with the proportion gametocyte-positive infections and gametocyte density. Parasite densities were log10 transformed for all calculations. To correct for imperfect detection of gametocytes and include low-density infections without gametocytes detected in multivariable models, +0.1 was added to all gametocyte density values prior to log10-transformation. For multivariable analyses, individuals were grouped into age classes ≤6, >6–12, >12–20, and >20 years. Survey and age group were included as fixed effects. The ratio of pf525 or pv525 transcripts per parasite genome was assessed, representing the proportion of gametocytes among all parasites. Infections (*Pf*: n = 12, *Pv*: n = 5) with densities at the technical limit of detection of 0.25 copies/μL blood (i.e. 1 DNA/RNA template per PCR) were excluded from correlation analysis as quantification is imprecise at very low densities, and including them at a set density of 0.25 copies/μL would artificially increase correlation. All data is available in supplementary file S1 Data.

Results

Prevalence and parasite density

Prevalence of *P. falciparum* by qPCR ranged from 0.14% (Solomon Islands, 5/3501) to 18.5% (PNG 2010, 385/2083), and *P. vivax* prevalence from 3.3% (Thailand, 144/4309) to 19.7% (PNG 2014, 496/2517) (Table 2 and Fig 1). Light microscopy (LM) detected 27.9–80.0% of *P. falciparum*, and 13.3–51.8% of *P. vivax* infections positive by qPCR (Table 2). Mean parasite densities of both species differed significantly between surveys (one-way ANOVA *P* < 0.0001), with no clear trend between population parasite prevalence and mean parasite densities (Table 2 and Fig 1). In each survey, 80.0–94.5% of all *P. falciparum* infections and 80.0–97.5%
of all \textit{P. vivax} infections (by qPCR) were asymptomatic, i.e., not accompanied by measured or reported febrile illness in the preceding 48 hours.

**Gametocyte carriage**

By \textit{pfs}25 RT-qPCR, the proportion of gametocyte-positive infections differed significantly between surveys (Chi-Square test, \(P<0.001\), Table 2). 93.75\% (15/16), 80.0\% (4/5), and 70.8\% (34/48) of \textit{P. falciparum} qPCR-positive individuals carried gametocytes in Brazil, Solomon Islands and Thailand, respectively, and 42.9\% and 60.5\% in PNG 2014 and PNG 2010, respectively. This resulted in a population \textit{P. falciparum} gametocyte prevalence ranging from 0.11\% in Solomon Islands to 11.2\% in PNG 2010 (Table 2). In univariate analysis across all surveys, no correlation between \textit{P. falciparum} DNA copy number and \textit{pfs}25 copy numbers was observed (gametocyte-positive infections above technical limit of detection included, \(n = 379\), \(R^2 = 0.013\), \(P = 0.804\), Fig 2A). However, each 10-fold increase in DNA copy number increased the odds to detect \textit{P. falciparum} gametocytes 1.59-fold (all infections included, \(n = 676\), \(P<0.001\)).

By \textit{pvs}25 RT-qPCR, \textit{P. vivax} gametocytes were detected in 22.6\% and 23.5\% of infected individuals in PNG 2014 and Solomon Islands, and in 48.9\%, 53.7\%, and 71.5\% of infections

| Table 2. Parasite and gametocyte prevalence by LM and qPCR and RT-qPCR, and parasite densities by qPCR. |
|---------------------------------------------------------------|
| Number of participants | PNG 2010 | PNG 2014 | Solomon Is. | Thailand | Brazil |
|-------------------------|----------|----------|-------------|-----------|--------|
| \textit{P. falciparum} |           |          |             |           |        |
| Prevalence by LM\(^1\) | 7.44\% (155/2083) | 2.74\% (69/2513) | 0.11\% (4/3501) | NA | 0.55\% (11/2010) |
| Prevalence by PCR       | 18.48\% (385/2083) | 8.98\% (226/2517) | 0.14\% (5/3501) | 1.11\% (48/4309) | 0.39\% (16/4083) |
| qPCR copies/\textmu L [CI95] | 26.0 [18.7–36.0] | 16.4 [11.4–23.0] | 7.7 [0.9–63.8] | 5.6 [2.4–13.5] | 30.7 [6.4–147.1] |
| Proportion LM positive | 37.40\% (144/385) | 27.88\% (63/226) | 80.00\% (4/5) | NA | 63.64\% (7/11) |
| Infected + febrile/reported fever | 12.77\% (48/376) | 5.48\% (12/219) | 20.0\% (1/5) | 8.7\% (4/46) | 16.67\% (2/12) |
| Gametocyte prevalence by RT-qPCR | 11.19\% (233/2083) | 3.85\% (97/2517) | 0.11\% (4/3501) | 0.86\% (37/4309) | 0.37\% (15/4083) |
| Proportion gametocyte positive\(^2\) | 60.52\% (233/385) | 42.92\% (97/226) | 80.0\% (4/5) | 70.83\% (34/48) | 93.75\% (15/16) |
| Proportion gametocyte carriers LM positive\(^3\) | 54.51\% | 37.11\% | 100.00\% | NA | 78.57\% |
| \textit{P. vivax} |           |          |             |           |        |
| Prevalence by LM\(^1\) | 7.00\% (146/2083) | 2.70\% (68/2513) | 3.63\% (127/3501) | NA | 1.79\% (36/2010) |
| Prevalence by PCR       | 12.96\% (270/2083) | 19.71\% (496/2517) | 13.37\% (468/3501) | 3.34\% (144/4309) | 3.82\% (156/4083) |
| qPCR copies/\textmu L [CI95] | 4.8 [3.98–5.9] | 2.0 [1.7–2.4] | 1.1 [1.0–1.3] | 2.2 [1.5–3.1] | 10.0 [6.7–14.7] |
| Proportion LM positive | 51.85\% (140/270) | 13.31\% (66496) | 27.14\% (127/468) | NA | 37.21\% (32/86) |
| Infected + febrile/reported fever | 8.27\% (22/266) | 2.52\% (12/477) | 20.0\% (93/465) | 8.51 (12/141) | 17.42\% (27/155) |
| Gametocyte prevalence by RT-qPCR | 6.34\% (132/2083) | 4.45\% (112/2517) | 3.14\% (110/3501) | 2.39\% (103/4309) | 2.03\% (83/4083) |
| Proportion gametocyte positive\(^2\) | 48.89\% (132/270) | 22.58\% (112/496) | 23.5\% (110/468) | 71.53\% (103/144) | 53.21\% (83/156) |
| Proportion gametocyte carriers LM positive\(^3\) | 84.09\% | 39.64\% | 49.09\% | NA | 58.14\% |
| \textit{P. falciparum}/\textit{P. vivax} co-infections\(^4\) |           |          |             |           |        |
| Prevalence by LM | 0.72\% (15/2083) | 0.32\% (8/2513) | 0\% (0/3501) | NA | 0\% (0/2010) |
| Prevalence by qPCR | 3.90\% (81/2083) | 1.63\% (41/2517) | 0.14\% (5/3501) | 0.26\% (11/4309) | 0.02\% (1/4083) |

NA = not available
\(^1\) Positive by LM for asexuals and/or gametocytes. In Brazil, LM was conducted only on a subset of \(n = 2010\) samples.
\(^2\) Proportion of qPCR positive samples with gametocytes detected by RT-qPCR
\(^3\) Proportion of study subjects positive for gametocytes by RT-qPCR with asexual parasites and/or gametocytes detected by LM
\(^4\) Data in the Table for \textit{P. falciparum} and \textit{P. vivax} includes co-infections with the respective other species. Prevalence of co-infections represents co-infections among all individuals sampled (not among those positive by either species).

https://doi.org/10.1371/journal.pntd.0009672.t002
This resulted in a population *P. vivax* gametocyte prevalence of 2.0–4.3%. *P. vivax* parasite and gametocyte densities were strongly correlated (n = 415, $R^2 = 0.69, P < 0.0001$, Fig 2B), and a 10-fold increase in DNA copy number increased the odds of detecting *P. vivax* gametocyte by 2.91-fold (n = 1501, $P < 0.001$).

To assess whether the proportion of gametocytes among all blood-stage parasites differs between infections of different parasite density, the number of *pfs25* or *pvs25* transcripts per *P. falciparum* or *P. vivax* genome among gametocyte-positive samples was plotted (Fig 3A and 3B, infections with ≤5 DNA copies excluded). For *P. falciparum*, a 0.39-fold decrease in the proportion gametocytes per 10-fold increase in parasite density was observed (n = 255, $P < 0.001$), while no significant change was observed for *P. vivax* (n = 243, 1.19-fold increase per 10-fold increase in parasite density, $P = 0.057$). A febrile episode and/or antimalarial treatment in the preceding 2 weeks increased the proportion gametocytes significantly for *P.*
Multivariate risk factors of gametocyte positivity and density

In multivariate analysis across all surveys, parasite density was a strong predictor for the probability that a sample was gametocyte-positive (Table 3). Each 10-fold increase in \textit{P. falciparum} parasite density resulted in a 1.59-fold increase in the odds of gametocyte positivity, and in a 1.9-fold increase in gametocyte densities (Table 3). The correlation was much stronger for \textit{P. vivax}, where each 10-fold increase in parasite density resulted in a 3.15-fold increase in the odds of gametocyte positivity and a 3.9-fold increase in gametocyte density (Table 3). Concordance between \textit{P. vivax} genome and gametocyte density was even higher for infections above 5
DNA copies/uL (n = 373), with 9.0-fold more gametocytes per 10-fold increase in genome density.

Among *P. falciparum* positive individuals, the odds to detect gametocytes was 54% lower (*P*<0.0001) and gametocyte densities were 69% lower (*P*<0.0001) in individuals co-infected with *P. vivax*. Reported malaria or anti-malarial treatment in the past 2 weeks was associated with higher *P. falciparum* gametocyte prevalence and densities (Table 3). Parasite densities strongly decreased with increasing age. Even when including parasite density as confounder, *P. falciparum* gametocyte positivity and density decreased with increasing age, i.e., gametocyte densities decrease to a greater extent than blood-stage parasitemia (Table 3). Among *P. vivax* positive individuals, gametocyte densities, but not positivity decreased with age (Table 3). Apart from DNA copy numbers, no other significant associations were observed.

Factors affecting gametocyte density were assessed independently in PNG 2010 (moderate-high transmission), PNG 2014 (recently decreased transmission), and Thailand and Brazil pooled (long-time low transmission) (Table 4). For PNG 2010 results were very similar to

### Table 3. Multivariate predictors of gametocyte positivity and density.

| Gametocyte positivity | *P. falciparum* (n = 647) | *P. vivax* (n = 1501) |
|-----------------------|---------------------------|------------------------|
|                       | OR | *P* | OR | *P* |
| log<sub>10</sub> Pf/Pv copies | 1.59 | <0.0001 | 3.15 | <0.0001 |
| PNG 2010 (reference)  | [Reference] |         | [Reference] |         |
| PNG 2014              | 0.55 |         | 0.40 |         |
| Solomon Is.           | 8.54 | <0.0001 | 0.62 | <0.0001 |
| Thailand              | 2.99 |         | 5.60 |         |
| Brazil                | 11.03 |         | 0.65 |         |
| age: 0–6 y (reference) | [Reference] |         |         |         |
| age: >6–12 y          | 1.13 |         |         |         |
| age: >12–20 y         | 0.56 | 0.0006 |         |         |
| age: >20 y            | 0.47 |         |         |         |
| *P. vivax* PCR positive | 0.41 | <0.0001 |         | <0.0001 |
| Reported malaria last 2 weeks | 2.28 | 0.032 |         |         |

| Gametocyte density | *P. falciparum* (n = 642) | *P. vivax* (n = 1041) |
|--------------------|---------------------------|------------------------|
|                     | Coef. | *P* | Coef. | *P* |
| log<sub>10</sub> Pf/Pv copies | 0.20 | <0.0001 | 0.59 | <0.0001 |
| PNG 2010 (reference)  | [Reference] |         | [Reference] |         |
| PNG 2014              | -0.44 |         | -0.08 |         |
| Thailand              | 0.58 | <0.0001 | 0.83 | <0.0001 |
| Brazil                | 1.73 |         | 0.02 |         |
| age: 0–6 y (reference) | [Reference] |         | [Reference] |         |
| age: >6–12 y          | -0.33 |         | -0.07 |         |
| age: >12–20 y         | -0.76 | <0.0001 | -0.18 | 0.0167 |
| age: >20 y            | -0.82 |         | -0.20 |         |
| *P. vivax* PCR positive | -0.51 | <0.0001 |         | <0.0001 |
| Reported malaria last 2 weeks | 0.82 |         | 0.82 |         |

(A) Gametocyte positivity, (B) gametocyte density. Additional factors tested, but not found to be associated, included fever measured at the day of survey or reported from the past 2 days, and reported malaria or antimalarial treatment in the past 2 weeks. In each subgroup, only variables with a significant effect (*P*<0.05) on gametocyte positivity or densities are shown.

https://doi.org/10.1371/journal.pntd.0009672.t003
pooled data from all surveys. In PNG 2014, for *P. falciparum*, gametocyte densities decreased with age and were lower in individuals co-infected with *P. vivax*, but were not significantly associated with parasite density (*P* = 0.085). In Thailand and Brazil, for both species, and for *P. vivax* in PNG 2014, no other factors than parasite density were significantly associated with gametocyte density.

As a result of the correlation of parasite and gametocyte density and the decreasing parasite densities with age in both PNG surveys, for both species the majority of individuals with detectable gametocytes were children. 48–78% of gametocyte carriers were <12 years (Fig 4). In contrast, 65–67% of gametocyte carriers were >20 years in Thailand and Brazil.

When including parasite density in multivariable analysis to correct for differences in mean density among surveys, the proportion gametocyte positive infections, and gametocyte densities of both species differed significantly between surveys (*P*<0.001, Table 3). The probability to detect gametocytes increased in all surveys with increasing genome density. An interaction analysis did not reveal a significant difference of this increase between surveys (*Pf*: n = 676, *P* = 0.471; *Pv*: n = 1501, *P* = 0.512). Likewise, *P. falciparum* and *P. vivax* gametocyte densities were not affected by an interaction between DNA copies and survey (*Pf*: n = 375, *P* = 0.877, *Pv*: n = 415, *P* = 0.132).

Hemoglobin measurements and multiplicity of infection data were available from the surveys in PNG and Solomon Islands; neither affected gametocyte positivity or density in multivariate analysis (S2 and S3 Tables).

### Table 4. Multivariable predictors of *P. falciparum* and *P. vivax* gametocyte density under different transmission scenarios.

#### A) *P. falciparum*

|                      | PNG 2010 (n = 364) |                  |                  |                  |                  |                  |                  |
|----------------------|--------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                      | Coef.              | *P*             | Coef.           | *P*             | Coef.           | *P*             | Coef.           | *P*             |
| log₁₀ *Pf* copies    | 0.34               | <0.001          | 0.37            | 0.015           | 0.37            | 0.015           |
| age: 0–6 y (reference)| [Reference]        |                 |                 |                 |                 |                 |
| age: >6–12 y         | -0.37              | -0.42           |                 |                 |                 |                 |
| age: >12–20 y        | -0.71              | 0.002           | -1.01           | <0.001          |                 |                 |
| age: >20 y           | -0.84              | -1.03           |                 |                 |                 |                 |
| *Pv* PCR positive    | -0.51              | 0.002           | -0.81           | <0.001          |                 |                 |
| Reported malaria last 2 weeks | 1.13       |                 | <0.001          |                 |                 |                 |

#### B) *P. vivax*

|                      | PNG 2010 (n = 252) |                  |                  |                  |                  |                  |                  |
|----------------------|--------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                      | Coef.              | *P*             | Coef.           | *P*             | Coef.           | *P*             | Coef.           | *P*             |
| log₁₀ *Pv* copies    | 0.63               | <0.001          | 0.47            | <0.001          | 0.62            | <0.001          |
| age: 0–6 y (reference)| [Reference]        |                 |                 |                 |                 |                 |
| age: >6–12 y         | -0.25              |                 |                 |                 |                 |                 |
| age: >12–20 y        | -0.42              | 0.0009          |                 |                 |                 |                 |
| age: >20 y           | -0.52              |                 |                 |                 |                 |                 |
| *Pf* PCR positive    |                    |                 |                 |                 |                 |                 |
| Reported malaria last 2 weeks |          |                 |                 |                 |                 |                 |

Multivariable predictors of *P. falciparum* and *P. vivax* gametocyte density in PNG 2010 (intermediate transmission), PNG 2014 (recent reduction in transmission), and Thailand and Brazil pooled (extended period of low transmission). In each subgroup, only variables with a significant effect (*P*<0.05) on gametocyte densities were included.

https://doi.org/10.1371/journal.pntd.0009672.t004
Ability of light microscopy to diagnose gametocyte carriers

Restricting the analysis to sites where LM was conducted (PNG, Solomon Islands, Brazil, n = 10,107), *P. falciparum* gametocytes were detected by RT-qPCR in 80.2% (178/222) of LM-positive individuals. Among LM-negative individuals, gametocytes were detected in 40.2% (170/422) of individuals. *Pf*25 transcript densities were nearly 10-fold higher in LM-positive individuals (33.40 vs. 4.27 transcripts/μL, \( P = 0.001 \)). The ability of LM to detect *P. falciparum* gametocyte carriers differed significantly between surveys (\( P < 0.001 \), Table 2), and appeared to be particularly high when prevalence was low. For example, 4/4, and 7/10 *P. falciparum* gametocyte carriers were LM-positive in Solomon Islands and Brazil, respectively (Table 2).

*P. vivax* gametocytes were detected by RT-qPCR in 64.9% (235/362) of LM-positive individuals, but in only 16.7% (163/976) of LM-negative individuals. Mean gametocyte densities in LM-positive individuals were almost twice as high compared to LM-negative individuals (6.02 vs. 3.44 transcripts/μL, \( P < 0.001 \)). Thus, approximately 50% of *P. falciparum* and 59% of *P. vivax* gametocyte carriers were positive by light microscopy.

Discussion

We observed substantial differences in the proportion of *P. falciparum* and *P. vivax* infections carrying detectable gametocytes in 5 cross-sectional surveys representing distinct malaria-epidemiological contexts. The proportion of gametocyte-positive infections in community surveys is heavily impacted by the sensitivity of the assays used for parasite and gametocyte detection [39]. The use of the identical methodology and external reference standards for all surveys allowed, for the first time, direct comparisons between regions of different transmission intensity.
Blood-stage parasite densities were a strong predictor for gametocyte positivity and could largely explain differences between surveys. In most surveys, the majority of gametocyte carriers of both Plasmodium species (as determined by RT-qPCR) were positive by LM for asexual parasites. A lower proportion of gametocyte carriers was LM-positive in Madang 2014 (both species) and Solomon Islands (P. vivax), where transmission had declined in the years prior to the surveys [28, 30], and parasite densities were very low. In these surveys, a large proportion of gametocyte carriers could not be diagnosed by microscopy. In contrast, in the sites where transmission had been reduced for longer (Brazil, P. falciparum in Solomon Islands), expert LM or other tools such as RDT remain sufficiently sensitive to identify the majority of gametocyte carriers.

Pronounced age trends in gametocyte carriage were evident in PNG and Solomon Islands. Prevalence of infection peaked in children or adolescents, and parasite densities decreased rapidly with increasing age, most likely due to the acquisition of immunity. As a result, the vast majority of gametocytes were detected in children below 6 years, especially for P. vivax. This contrasts findings from P. falciparum in Africa, where school-age children were proposed to contribute most to transmission densities [40, 41]. In moderate-high transmission settings and in regions of steep decline in transmission in recent years, gametocyte densities decreased even faster than parasite densities with age. Thus, changes in parasite prevalence and density with age might not appropriately reflect changes in transmission potential. In Brazil and Thailand, the risk of infection increased with increasing age, age trends of parasite densities were moderate, and as a result no age trends in gametocyte densities were evident.

Apart from parasite density, limited effects of transient factors on gametocyte densities were observed. Multiple clone infection or hemoglobin levels did not affect gametocyte carriage of either species. For P. vivax, a constant proportion of gametocytes among all parasites was observed irrespective of parasite density. In the case of P. falciparum, high proportions of gametocytes were observed in a subset of infections with low-to-moderate densities. The 2-week sequestration of developing P. falciparum gametocytes results in a temporal lag of peak gametocytemia following peak parasitemia [6]. Thus, infections with low parasite but high gametocyte densities might have experienced a recent wave of asexual parasitemia [42]. This is corroborated by the fact that self-reported febrile illness in the two weeks prior to sample collection resulted in higher gametocyte densities (Fig 3C and 3D). Conversion of a large proportion of all parasites into gametocytes when parasite densities drop to very low levels has also been described in a rodent malaria model [43]. Longitudinal studies with frequent sampling will be needed to assess how closely P. falciparum gametocyte density reflects parasite density in the preceding 2 weeks.

As an exception to the limited impact of transient factors, a lower proportion P. falciparum gametocyte positive infections and lower gametocyte densities were observed in individuals co-infected with P. vivax. It is not known whether co-infection results in an adjustment of the gametocyte conversion rate, or whether multi-species infection is a surrogate marker for higher exposure, and thus, higher levels of gametocyte-specific immunity.

Mosquito feeding assays have repeatedly shown a correlation between parasite density and infectivity. Few studies have included asymptomatic individuals and individuals negative by LM for gametocytes and asexual stages [44–50]. With few exceptions, e.g., one study on P. vivax infectivity in Brazil [48], individuals with either asexual parasites or gametocytes detected by LM were far more infective than submicroscopic infections (Fig 5). For P. vivax, in Thailand a steep increase in infectivity was found at densities (by LM) of 10–100 parasites/μL, with little effect if densities increased further [47]. This density closely matches the limit of detection of expert LM. The finding in the present study of a majority of gametocytes
concentrated in LM-positive individuals, together with the results from mosquito-feeding studies, corroborate that LM-positive individuals likely are the main infectious reservoir.

Studies assessing gametocyte densities and infectivity over time will be required to determine what proportion of low-density infections will rise in density and become highly infective. The importance of male gametocyte densities to predict infectivity is increasingly recognized. In low density infections, male gametocytes might be the limiting factor for onward transmission [51]. Measuring male gametocyte densities in addition to female densities, as measured by \( pfs25 \) and \( pvs25 \) RT-qPCR, is expected to allow for better predictions of infectivity [51]. Due to the non-linear relationship between gametocyte density and infectivity, age trends in gametocyte density might not fully reflect infectivity. Lastly, while higher gametocyte densities result in increased oocyst numbers in mosquitos [51], it is unclear whether oocyst numbers have an effect on the efficiency of onward transmission.

Even after correcting for different mean parasite densities between surveys, a higher proportion gametocyte positive samples were found for both species in Thailand and for \( P. falciparum \) in Brazil and Solomon Islands compared to PNG. Recent malaria control activities have resulted in significant changes in vector composition and biting behavior in the study sites [52, 53]. A reduction in the number of mosquito bites or shift towards a less competent vector might select for parasites with higher gametocyte conversion rates. Such a selection has been suggested by recent genome and transcriptome studies. Expression of the AP2-G transcription factor and additional epigenetic factors involved in gametocytogenesis were adjusted to transmission levels in \( P. falciparum \) populations in East Africa [54], and the \( \textit{gametocyte development gene 1 (gad1)} \), which is essential for early gametocyte development, was found to be under strong selection in \( P. falciparum \) populations in regions of different endemicity in West Africa [55]. In Cambodia, control efforts resulted in strong selection of the AP2-G homolog in \( P. vivax \) [56]. The present study, for the first time, found differences in the proportion of gametocytes among all parasites between regions of different transmission intensity.

**Conclusions**

The probability to detect gametocytes was closely correlated to blood-stage parasitemia in different transmission settings. The vast majority of all infections with high gametocyte densities (as determined by RT-qPCR) could be diagnosed by microscopy. Pronounced age trends of gametocyte carriage in areas of moderate to high transmission was observed. Even though the contribution to transmission is influenced by vector exposure and transmission blocking...
immunity in addition to gametocyte prevalence and density, the age trends suggest that interventions to reduce transmission will have the greatest effect when targeted towards children. In contrast, in order to achieve elimination in low transmission settings individuals of all ages need to be protected from vector contact, which is often not the case [57].

Supporting information
S1 Data. Database.
(TXT)

S1 Table. Distribution of study participants by age group and by gender in each survey.
(DOCX)

S2 Table. Effect of hemoglobin levels on gametocyte positivity and density.
(DOCX)

S3 Table. Effect of multiple clone infection on gametocyte positivity and density.
(DOCX)

Acknowledgments
We would like to thank all individuals who participated in the studies, the field teams who conducted the sample collection, and the molecular and microscopy teams. We thank Nicolas Senn for his support of the 2010 PNG survey.

Author Contributions
Conceptualization: Cristian Koepfli, James Kazura, Quique Bassat, Ingrid Felger, Jetsumon Sattabongkot, Leanne J. Robinson, Marcus Lacerda, Ivo Mueller.

Data curation: Cristian Koepfli, Wang Nguitragool, Anne Cristine Gomes de Almeida.

Formal analysis: Cristian Koepfli, Ivo Mueller.

Funding acquisition: James Kazura, Ingrid Felger, Jetsumon Sattabongkot, Leanne J. Robinson, Marcus Lacerda, Ivo Mueller.

Investigation: Cristian Koepfli, Wang Nguitragool, Anne Cristine Gomes de Almeida, Andrea Kuehn, Andrea Waltmann, Eline Kattenberg, Maria Ome-Kaius, Patricia Rarau, Wuelton Monteiro, Andrew W. Darcy, Lyndes Wini, Jetsumon Sattabongkot, Leanne J. Robinson, Marcus Lacerda, Ivo Mueller.

Methodology: Thomas Obadia, Ingrid Felger, Ivo Mueller.

Project administration: Cristian Koepfli, James Kazura, Wuelton Monteiro, Quique Bassat, Leanne J. Robinson, Ivo Mueller.

Supervision: Jetsumon Sattabongkot, Leanne J. Robinson, Marcus Lacerda, Ivo Mueller.

Validation: Cristian Koepfli, Thomas Obadia.

Visualization: Cristian Koepfli.

Writing – original draft: Cristian Koepfli.

Writing – review & editing: Cristian Koepfli, Wang Nguitragool, Anne Cristine Gomes de Almeida, Andrea Kuehn, Andrea Waltmann, Eline Kattenberg, Maria Ome-Kaius, Patricia Rarau, Thomas Obadia, James Kazura, Wuelton Monteiro, Andrew W. Darcy, Lyndes
Wini, Quique Bassat, Ingrid Felger, Jetsumon Sattabongkot, Leanne J. Robinson, Marcus Lacerda, Ivo Mueller.

References

1. Pluess B, Tanser FC, Lengeler C, Sharp BL. Indoor residual spraying for preventing malaria. Cochrane Database Syst Rev. 2010;(4):CD006657. Epub 2010/04/16. https://doi.org/10.1002/14651858.CD006657.pub2 PMID: 20393950; PubMed Central PMCID: PMC6532743.

2. Sutanto I, Kosasih A, Elyazar IRF, Simanjuntak DR, Larasati TA, Dahlan MS, et al. Negligible Impact of Mass Screening and Treatment on Mesoendemic Malaria Transmission at West Timor in Eastern Indonesia: A Cluster-Randomized Trial. Clin Infect Dis. 2018; 67(9):1364–72. Epub 2018/03/27. https://doi.org/10.1093/cid/ciy231 PMID: 29579195; PubMed Central PMCID: PMC6186863.

3. von Seidlein L, Peto TJ, Landier J, Nguyen TN, Tripura R, Phommasone K, et al. The impact of targeted malaria elimination with mass drug administrations on falciparum malaria in Southeast Asia: A cluster randomised trial. PLoS Med. 2019; 16(2):e1002745. Epub 2019/02/16. https://doi.org/10.1371/journal.pmed.1002745 PMID: 30768615; PubMed Central PMCID: PMC6377128.

4. Olotu A, Fegan G, Wambua J, Nyangweso G, Leach A, Lievens M, et al. Seven-Year Efficacy of RTS, S/AS01 Malaria Vaccine among Young African Children. N Engl J Med. 2016; 374(26):2519–29. Epub 2016/06/30. https://doi.org/10.1056/NEJMoa1515257 PMID: 27355532; PubMed Central PMCID: PMC4962898.

5. Slater HC, Foy BD, Kobylinski K, Chacour C, Watson OJ, Hellewell J, et al. Ivermectin as a novel complementary malaria control tool to reduce incidence and prevalence: a modelling study. Lancet Infect Dis. 2020. Epub 2020/01/18. https://doi.org/10.1016/S1473-3099(19)30633-4 PMID: 31948767.

6. Bousema T, Drakeley C. Epidemiology and infectivity of Plasmodium falciparum and Plasmodium vivax gametocytes in relation to malaria control and elimination. Clin Microbiol Rev. 2011; 24(2):377–410. Epub 2011/04/13. https://doi.org/10.1128/CMR.00051-10 PMID: 21482730; PubMed Central PMCID: PMC3122489.

7. Smalley ME, Abdalla S, Brown J. The distribution of Plasmodium falciparum in the peripheral blood and bone marrow of Gambian children. Trans R Soc Trop Med Hyg. 1981; 75(1):103–5. Epub 1981/01/01. https://doi.org/10.1016/0035-9203 (81)90019-5 PMID: 7022784.

8. Farfour E, Charlotte F, Settegrana C, Miyara M, Buffet P. The extravascular compartment of the bone marrow: a niche for Plasmodium falciparum gametocyte maturation? Malar J. 2012; 11:285. Epub 2012/08/22. https://doi.org/10.1186/1475-2875-11-285 PMID: 22905863; PubMed Central PMCID: PMC3472181.

9. Stepniewska K, Price RN, Sutherland CJ, Drakeley CJ, von Seidelein L, Nosten F, et al. Plasmodium falciparum gametocyte dynamics in areas of different malaria endemicity. Malar J. 2008; 7:249. Epub 2008/12/06. https://doi.org/10.1186/1475-2875-7-249 PMID: 19055751; PubMed Central PMCID: PMC2635378.

10. Eichner M, Diedner HH, Molineaux L, Collins WE, Jeffery GM, Dietz K. Genesis, sequestration and survival of Plasmodium falciparum gametocytes: parameter estimates from fitting a model to malaria therapy data. Trans R Soc Trop Med Hyg. 2001; 95(5):497–501. Epub 2001/11/15. https://doi.org/10.1016/s0035-9203(01)90016-1 PMID: 11706658.

11. McCarthy JS, Ruckle T, Djeriou E, Cantalloube C, Ter-Minassian D, Baker M, et al. A Phase II pilot trial to evaluate safety and efficacy of ferroquine against early Plasmodium falciparum in an induced blood-stage malaria infection study. Malar J. 2016; 15:469. Epub 2016/09/15. https://doi.org/10.1186/s12936-016-1511-3 PMID: 27624471; PubMed Central PMCID: PMC5022189.

12. Bousema T, Okell L, Shekalahge S, Griffin JT, Omar S, Sawa P, et al. Revisiting the circulation time of Plasmodium falciparum gametocytes: molecular detection methods to estimate the duration of gametocyte carriage and the effect of gametocytocidal drugs. Malar J. 2010; 9:136. Epub 2010/05/26. https://doi.org/10.1186/1475-2875-9-136 PMID: 20497536; PubMed Central PMCID: PMC2881938.

13. Group WGS. Gametocyte carriage in uncomplicated Plasmodium falciparum malaria following treatment with artemisinin combination therapy: a systematic review and meta-analysis of individual patient data. BMC Med. 2016; 14:79. Epub 2016/05/26. https://doi.org/10.1186/s12936-016-0621-7 PMID: 27221542; PubMed Central PMCID: PMC4679753.

14. Stepniewska K, Humphreys GS, Goncalves BP, Craig E, Gsoling R, Guerin PJ, et al. Efficacy of single dose primaquine with artemisinin combination therapy on P. falciparum gametocytes and transmission: A WWARN individual patient meta-analysis. J Infect Dis. 2020. Epub 2020/08/12. https://doi.org/10.1093/infdis/jiaa498 PMID: 32778675.
15. Sutanto I, Suprijanto S, Kosasih A, Dahlan MS, Syafruddin D, Kusriastuti R, et al. The effect of primaquine on gametocyte development and clearance in the treatment of uncomplicated falciparum malaria with dihydroartemisinin-piperaquine in South sumatra, Western Indonesia: an open-label, randomized, controlled trial. Clin Infect Dis. 2013; 56(5):685–83. Epub 2012/11/24. https://doi.org/10.1093/cid/cis959 PMID: 23175563.

16. Okebe J, Bousema T, Affara M, Di Tanna GL, Dabira E, Gaye A, et al. The Gametocytocidal Efficacy of Different Single Doses of Primaquine with Dihydroartemisinin-piperaquine in Asymptomatic Parasite Carriers in The Gambia: A Randomized Controlled Trial. EBioMedicine. 2016; 13:348–55. Epub 2016/11/09. https://doi.org/10.1016/j.ebiom.2016.10.032 PMID: 27825738; PubMed Central PMCID: PMC5264436.

17. Griffin P, Pasay C, Elliott S, Sekuloski S, Sikulu M, Hugo L, et al. Safety and Reproducibility of a Clinical Trial System Using Induced Blood Stage Plasmodium vivax Infection and Its Potential as a Model to Evaluate Malaria Transmission. PLoS Negl Trop Dis. 2016; 10(12):e0005139. Epub 2016/12/09. https://doi.org/10.1371/journal.pntd.0005139 PMID: 27930652; PubMed Central PMCID: PMC5145139 following competing interests: PG and SE are employed by Q-Pharm Pty Ltd; CO and Ki are employed by PATH MVI.

18. McKenzie FE, Jeffery GM, Collins WE. Plasmodium vivax blood-stage dynamics. J Parasitol. 2002; 88(3):521–35. Epub 2002/07/09. https://doi.org/10.1645/0022-3395(2002)088[0521:PVBSD]2.0.CO;2 PMID: 12099421; PubMed Central PMCID: PMC2504326.

19. Hasugian AR, Purba HL, Kenangalem E, Wuwung RM, Ebsworth EP, Maristela R, et al. Dihydroartemisinin-piperaquine versus artemunate-amodiaquine: superior efficacy and posttreatment prophylaxis against multidrug-resistant Plasmodium falciparum and Plasmodium vivax malaria. Clin Infect Dis. 2007; 44(8):1067–74. Epub 2007/03/17. https://doi.org/10.1086/512677 PMID: 17366451; PubMed Central PMCID: PMC5232501.

20. Grigg MJ, William T, Menon J, Barber BE, Wilkes CS, Rajahram GS, et al. Efficacy of Artesunate-mefloquine for Chloroquine-resistant Plasmodium vivax malaria in Malaysia: An Open-label, Randomized, Controlled Trial. Clin Infect Dis. 2016; 62(11):1403–11. Epub 2016/04/24. https://doi.org/10.1093/cid/ciw121 PMID: 27107287; PubMed Central PMCID: PMC4872287.

21. Beyene HB, Beyene MB, Ebstie YA, Desalegn Z. Efficacy of Chloroquine for the Treatment of Vivax malaria in Northwest Ethiopia. PLoS One. 2016; 11(8):e0161483. Epub 2016/09/01. https://doi.org/10.1371/journal.pone.0161483 PMID: 27579480; PubMed Central PMCID:PMC5007045.

22. Douglas NM, Simpson JA, Phyo AP, Siwastoro H, Hasugian AR, Kenangalem E, et al. Gametocyte dynamics and the role of drugs in reducing the transmission potential of Plasmodium vivax. J Infect Dis. 2013; 208(5):801–12. Epub 2013/06/15. https://doi.org/10.1093/infdis/jit261 PMID: 23768527; PubMed Central PMCID: PMC3733516.

23. Cheng Q, Cunningham J, Gatton ML. Systematic review of sub-microscopic P. vivax infections: prevalence and determining factors. PLoS Negl Trop Dis. 2015; 9(1):e3413. Epub 2015/01/09. https://doi.org/10.1371/journal.pntd.0003413 PMID: 25569135; PubMed Central PMCID: PMC4288718.

24. Okeel LC, Ghani AC, Lyons E, Drakeley CJ. Submicroscopic infection in Plasmodium falciparum-endemic populations: a systematic review and meta-analysis. J Infect Dis. 2009; 200(10):1509–17. Epub 2009/10/24. https://doi.org/10.1086/644781 PMID: 19848588.

25. Schneider P, Schoone G, Schallig H, Verhage D, Teigt D, Eling W, et al. Quantification of Plasmodium falciparum gametocytes in differential stages of development by quantitative nucleic acid sequence-based amplification. Mol Biochem Parasitol. 2004; 137(1):35–41. https://doi.org/10.1016/j.molbiopara.2004.03.018 PMID: 15279949.

26. Babiker HA, Abdel-Wahab A, Ahmed S, Sulei man S, Ranford-Cartwright L, Carter R, et al. Detection of low level Plasmodium falciparum gametocytes using reverse transcriptase polymerase chain reaction. Mol Biochem Parasitol. 1998; 95(1):143–8. https://doi.org/10.1016/s0166-6851(98)00178-3 PMID: 10215031.

27. Koepfli C, Robinson LJ, Rarau P, Salib M, Sambale N, Wampfier R, et al. Blood-Stage Parasitaemia and Age Determine Plasmodium falciparum and P. vivax Gametocytaemia in Papua New Guinea. PLoS One. 2015; 10(5):e0126747. Epub 2015/05/23. https://doi.org/10.1371/journal.pone.0126747 PMID: 25996916; PubMed Central PMCID: PMC4440770.

28. Koepfli C, Ome-Kiausi M, Jally S, Malau E, Maripal S, Ginny J, et al. Sustained Malaria Control Over an 8-Year Period in Papua New Guinea: The Challenge of Low-Density Asymptomatic Plasmodium Infections. J Infect Dis. 2017; 216(11):1434–43. Epub 2017/10/14. https://doi.org/10.1093/infdis/jix507 PMID: 29029179; PubMed Central PMCID: PMC5853328.

29. Nguitragool W, Mueller I, Kumphilat C, Saeseu T, Bantuchai S, Yor saeng R, et al. Very high carriage of gametocytes in asymptomatic low-density Plasmodium falciparum and P. vivax infections in western Thailand. Parasit Vectors. 2017; 10(1):512. Epub 2017/10/27. https://doi.org/10.1186/s13071-017-2407-y PMID: 29065910; PubMed Central PMCID: PMC565986.
PLOS NEGLECTED TROPICAL DISEASES

Asymptomatic *P. falciparum* and *P. vivax* gametocyte reservoir

30. Waltmann A, Darcy AW, Harris I, Koepfli C, Lodo J, Vahi V, et al. High Rates of Asymptomatic, Sub-microscopic Plasmodium vivax Infection and Disappearing Plasmodium falciparum Malaria in an Area of Low Transmission in Solomon Islands. PLoS Negl Trop Dis. 2015; 9(5):e0003758. Epub 2015/05/23. https://doi.org/10.1371/journal.pntd.0003758 PMID: 25996619; PubMed Central PMCID: PMC4440702.

31. Almeida ACG, Kuehn A, Castro AJM, Vitor-Silva S, Figueiredo EFG, Brasil LW, et al. High proportions of asymptomatic and submicroscopic Plasmodium vivax infections in a peri-urban area of low transmission in the Brazilian Amazon. Parasit Vectors. 2018; 11(1):194. Epub 2018/03/22. https://doi.org/10.1186/s13071-018-2787-7 PMID: 29558395; PubMed Central PMCID: PMC5859403.

32. Barbosa S, Gozze AB, Lima NF, Batista CL, Bastos Mda S, Nicolete VC, et al. Epidemiology of disappearing Plasmodium vivax malaria: a case study in rural Amazonia. PLoS Negl Trop Dis. 2014; 8(8): e3109. Epub 2014/08/29. https://doi.org/10.1371/journal.pntd.0003109 PMID: 25166263; PubMed Central PMCID: PMC4148206.

33. Rovira-Vallbona E, Contreras-Mancilla JJ, Ramirez R, Guzman-Guzman M, Carrasco-Escobar G, Llamos-Cuestas A, et al. Predominance of asymptomatic and sub-microscopic infections characterizes the Plasmodium gametocyte reservoir in the Peruvian Amazon. PLoS Negl Trop Dis. 2017; 11(7): e0005674. Epub 2017/07/04. https://doi.org/10.1371/journal.pntd.0005674 PMID: 28671944; PubMed Central PMCID: PMC5510906.

34. Rosanas-Urgell A, Mueller D, Betuela I, Barnadas C, Iga J, Zimmermann PA, et al. Comparison of diagnostic methods for the detection and quantification of the four sympatric Plasmodium species in field samples from Papua New Guinea. Malar J. 2010; 9:361. Epub 2010/12/16. https://doi.org/10.1186/1475-2875-9-361 PMID: 21156052; PubMed Central PMCID: PMC3016373.

35. Wampfler R, Mwingira F, Javati S, Robinson L, Betuela I, Siba P, et al. Strategies for Detection of Plasmodium species Gametocytes. Plos One. 2013; 8(9): e76316. https://doi.org/10.1371/journal.pone.0076316 WOS:000325223900097. PMID: 24312682.

36. Koepfli C, Nguitragool W, Hofmann NE, Robinson L, Betuela I, Siba P, et al. Sensitive and accurate quantification of human malaria parasites using droplet digital PCR (ddPCR). Sci Rep. 2016; 6:39183. Epub 2016/12/17. https://doi.org/10.1038/srep39183 PMID: 27982132; PubMed Central PMCID: PMC5159915.

37. Falk N, Maire N, Sama W, Owusu-Agyei S, Smith T, Beck HP, et al. Comparison of PCR-RFLP and Genescan-based genotyping for analyzing infection dynamics of Plasmodium falciparum. Am J Trop Med Hyg. 2006; 74(6):944–50. Epub 2006/06/09. PMID: 16760501.

38. Koepfli C, Rodrigues PT, Antao T, Orjuela-Sanchez P, Van den Eede P, Gamboa D, et al. Plasmodium vivax Diversity and Population Structure across Four Continents. PLoS Negl Trop Dis. 2015; 9(6): e0003872. Epub 2015/07/01. https://doi.org/10.1371/journal.pntd.0003872 PMID: 26125189; PubMed Central PMCID: PMC4488360.

39. Koepfli C, Yan G. Plasmodium Gametocytes in Field Studies: Do We Measure Commitment to Transmission or Detectability? Trends Parasitol. 2018; 34(5):378–87. Epub 2018/03/17. https://doi.org/10.1016/j.pt.2018.02.009 PMID: 29544966; PubMed Central PMCID: PMC5924719.

40. Taylor SM, Sunner KM, Freedman B, Mangeni JN, Obala AA, Prudhomme O’Meara W. Direct Estimation of Sensitivity of Plasmodium falciparum Rapid Diagnostic Test for Active Case Detection in a High-Transmission Community Setting. Am J Trop Med Hyg. 2019; 101(6):1416–23. Epub 2019/11/02. https://doi.org/10.4269/ajtmh.19-0558 PMID: 31674301.

41. Coalson JE, Walldorf JA, Cohee LM, Ismail MAD, Mathanga D, Cordy RJ, et al. High prevalence of Plasmodium falciparum gametocyte infections in school-age children using molecular detection: patterns and predictors of risk from a cross-sectional study in southern Malawi. Malar J. 2016; 15(1):527. Epub 2016/11/05. https://doi.org/10.1186/s12936-016-1587-9 PMID: 27809907; PubMed Central PMCID: PMC5096312.

42. Collins KA, Wang CY, Adams M, Mitchell H, Rampton M, Elliott S, et al. A controlled human malaria infection model enabling evaluation of transmission-blocking interventions. J Clin Invest. 2018; 128(4):1551–62. Epub 2018/02/02. https://doi.org/10.1172/JCI99012 PMID: 29398961; PubMed Central PMCID: PMC5873858.

43. Schneider P, Greischar MA, Birget PLG, Repton C, Mideo N, Reece SE. Adaptive plasticity in the gametocyte conversion rate of malaria parasites. PLoS Pathog. 2018; 14(11):e1007371. Epub 2018/11/15. https://doi.org/10.1371/journal.ppat.1007371 PMID: 30427935; PubMed Central PMCID: PMC6261640.

44. Goncalves BP, Kapulu MC, Sawa P, Guelbeogo WM, Tiono AB, Grignard L, et al. Examining the human infectious reservoir for Plasmodium falciparum malaria in areas of differing transmission intensity. Nat Commun. 2017; 8(1):1133. Epub 2017/10/28. https://doi.org/10.1038/s41467-017-01270-4 PMID: 29074880; PubMed Central PMCID: PMC5658399.
45. Tadesse FG, Slater HC, Chai W, Teelen K, Lanke K, Belachew M, 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):1883–91. Epub 2018/01/06. https://doi.org/10.1093/cid/cix1123 PMID: 29304258.

46. Coleman RE, Kumpitak C, Ponlattav A, Maneechai N, Phunkitchar V, Rachapaew N, et al. Infectivity of asymptomatic Plasmodium-infected human populations to Anopheles dirus mosquitoes in western Thailand. J Med Entomol. 2004; 41(2):201–8. Epub 2004/04/06. https://doi.org/10.1603/0022-2585-41.2.201 PMID: 15061279.

47. Kiattibutr K, Roobsoong W, Sriwichai P, Suansomjit C, et al. Infectivity of symptomatic and asymptomatic Plasmodium vivax infections to a Southeast Asian vector, Anopheles dirus. Int J Parasitol. 2017; 47(2–3):163–70. Epub 2017/01/04. https://doi.org/10.1016/j.ijpara.2016.10.006 PMID: 28043858; PubMed Central PMCID: PMC5725394.

48. Martins-Campos KM, Kuehn A, Almeida A, Duarte APM, Sampaio VS, Rodriguez IC, et al. Infection of Anopheles aquasalis from symptomatic and asymptomatic Plasmodium vivax infections in Manaus, western Brazilian Amazon. Parasit Vectors. 2018; 11(1):288. Epub 2018/05/08. https://doi.org/10.1186/s13071-018-2749-0 PMID: 29728152; PubMed Central PMCID: PMC5935932.

49. Vallejo AF, Garcia J, Amado-Garavito AB, Arevalo-Herrera M, Herrera S. Plasmodium vivax gametocyte infectivity in sub-microscopic infections. Malar J. 2016; 15(1):48. https://doi.org/10.1186/s12936-016-0114-1 PMID: 26822406; PubMed Central PMCID: PMC4730736.

50. Ouedraogo AL, Goncalves BP, Gneme A, Wenger EA, Guelbeogo MW, Ouedraogo A, et al. Dynamics of the Human Infectious Reservoir for Malaria Determined by Mosquito Feeding Assays and Ultra-sensitive Malaria Diagnosis in Burkina Faso. J Infect Dis. 2016; 213(1):90–9. Epub 2015/07/05. https://doi.org/10.1093/infdis/jiv370 PMID: 26142435.

51. Bradley J, Stone W, Da DF, Morlais I, Dicko A, Cohuet A, et al. Predicting the likelihood and intensity of mosquito infection from sex specific Plasmodium falciparum gametocyte density. Elife. 2018; 7. Epub 2018/06/01. https://doi.org/10.7554/elife.34463 PMID: 29848446; PubMed Central PMCID: PMC6013255.

52. Sriwichai P, Karl S, Sumruayphol S, Kiattibutr K, Payakkapol A, et al. Evaluation of CDC light traps for mosquito surveillance in a malaria endemic area on the Thai-Myanmar border. Parasit Vectors. 2015; 8:636. https://doi.org/10.1186/s13071-015-1225-3 PMID: 26666683; PubMed Central PMCID: PMC4678759.

53. Gleave K, Cook D, Taylor MJ, Reimer LJ. Filarial infection influences mosquito behaviour and fecundity. Sci Rep. 2016; 6:36319. Epub 2016/11/01. https://doi.org/10.1038/srep36319 PMID: 27796352; PubMed Central PMCID: PMC5087081.

54. Rono MK, Nyonda MA, Simam JJ, Ngoi JM, Mok S, Kortok MM, et al. Adaptation of Plasmodium falciparum to its transmission environment. Nat Ecol Evol. 2018; 2(2):377–87. Epub 2017/12/20. https://doi.org/10.1038/s41559-017-0419-9 PMID: 29255304.

55. Mobegi VA, Duffy CW, Amambua-Ngwa A, Loua KM, Laman E, Nwakanma DC, et al. Genome-wide analysis of selection on the malaria parasite Plasmodium falciparum in West African populations of differing infection endemicity. Mol Biol Evol. 2014; 31(6):1490–9. https://doi.org/10.1093/molbev/msu106 PMID: 24644299; PubMed Central PMCID: PMC4032133.

56. Parobek CM, Lin JT, Saunders DL, Barnett EJ, Lon C, Lanteri CA, et al. Selective sweep suggests transcriptional regulation may underlie Plasmodium vivax resilience to malaria control measures in Cambodia. Proc Natl Acad Sci U S A. 2016. https://doi.org/10.1073/pnas.1608828113 PMID: 27911780.

57. Syafruddin D, Ashi PBS, Rozi IE, Permata DH, Nur Hidayati AP, Syahriani L, et al. Efficacy of a Spatial Repellent for Control of Malaria in Indonesia: A Cluster-Randomized Controlled Trial. Am J Trop Med Hyg. 2020; 103(1):344–58. Epub 2020/05/21. https://doi.org/10.4269/ajtmh.19-0554 PMID: 32431275; PubMed Central PMCID: PMC7356406.