Published in final edited form as:

Int J Parasitol. 2018 July ; 48(8): 601–610. doi:10.1016/j.ijpara.2018.02.004.

Infection of mosquitoes from *in vitro* cultivated *Plasmodium knowlesi* H strain

Jennifer S. Armistead[a,b,1], **Roberto R. Moraes Barros**[a,1], **Tyler J. Gibson**[a], **Whitney A. Kite**[a], **J. Patrick Mershon**[a], **Lynn Lambert**[c], **Sachy Orr-Gonzalez**[c], **Juliana M. Sá**[a], **John H. Adams**[b], and **Thomas E. Wellems**[a,2]

[a]Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

[b]Department of Global Health, College of Public Health, University of South Florida, Tampa, FL 33620

[c]Laboratory of Malaria Immunology and Vaccinology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Abstract

In vitro studies of sexual blood stages of the most fatal malaria species, *Plasmodium falciparum*, have revealed key processes by which gametocytes develop and transmit infection from humans to anopheline mosquitoes. However, most malaria cases outside sub-Saharan Africa are caused by other *Plasmodium* species, frequently *P. vivax* and *P. knowlesi*, a zoonotic parasite of macaque monkeys. Gametocytes of *P. vivax* and *P. knowlesi* exhibit distinct morphology, faster development, and shorter life span compared to gametocytes of *P. falciparum*, reflecting the evolutionary separation and biological differences of these species. Unlike *P. falciparum*, *P. vivax* cannot be cultivated *in vitro*, necessitating access to infected primates for laboratory studies. In contrast, *P. knowlesi* asexual stages have been successfully adapted to cultures in macaque and human red blood cells, but these stages have not been reported to produce gametocytes infective to mosquitoes. Here, we show that gametocyte production and sporadic, low-level mosquito infectivity of a *P. knowlesi* strain was not improved by application of a “crash” method commonly used to induce gametocytes in *P. falciparum* cultures. However, Percoll-gradient purified schizonts from this strain yielded highly synchronized populations that, in three of six experiments, produced infections at an average rate of 0.97 – 9.1 oocysts in *Anopheles dirus* mosquitoes. Oocyst counts were most abundant in mosquitoes that were fed from the synchronized cultures 36 h after schizont purification. Gametocytes in these cultures occurred at low-prevalence and were difficult to observe. Transcription from orthologs of *P. falciparum* gametocyte-specific markers did not correlate with infectivity of the *P. knowlesi* parasites to mosquitoes. Ability to infect

[2]To whom correspondence should be addressed. twellems@niaid.nih.gov. 12735 Twinbrook Parkway, Room 3E-10, Rockville, MD, 20852, USA. Phone: 301- 761-5085.

Author contributions: J.S.A. and R.R.M.B. conceived the research and designed experiments with J.M.S., J.H.A. and T.E.W.; J.S.A., R.R.M.B., T.J.G., J.M.S., L.L., S.O., W.K., and J.P.M. performed experiments; J.S.A., R.R.M.B., and T.J.G. analyzed data; J.S.A., R.R.M.B., T.J.G., J.M.S. and T.E.W. wrote the manuscript; all authors edited and approved the final report.

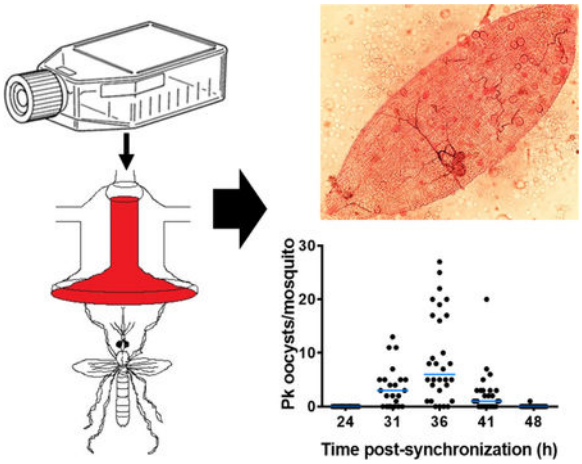
[1]J.S.A. and R.R.M.B. contributed equally to this work.

Footnotes

The authors declare no conflict of interest.

mosquitoes from *in vitro*-cultivated *P. knowlesi* will support research on the unique features of this emerging pathogen and facilitate comparative studies of transmission by the different human malarias.

Graphical Abstract



Keywords

malaria; zoonosis; gametocyte development; transmission

1. Introduction

Transmission of *Plasmodium* parasites that cause malaria requires maturation of infectious gametocytes within host red blood cells (RBC). When ingested in a bloodmeal by anopheline mosquitoes, gametocytes yield gametes in the insect midgut that initiate sexual reproduction required for the parasite life cycle. Continuous *in vitro* culture of the asexual blood stages of *Plasmodium falciparum* (Haynes et al., 1976; Trager and Jensen, 1976), the parasite species responsible for the deadliest human malaria, was a landmark in malaria research. Extension of these culture methods to generate sexual-stage gametocytes (Ifediba and Vanderberg, 1981) along with membrane-feeding techniques to infect mosquitoes (Graves, 1980; Ponnudurai et al., 1982), have facilitated discoveries of sexual commitment mechanisms (Brancucci et al., 2014; Coleman et al., 2014; Kafsack et al., 2014; Mantel et al., 2013; Regev-Rudzki et al., 2013), characterization of gametocyte-specific genes and processes (Meibalan and Marti, 2017), genetic crosses (Hayton et al., 2008; Walliker et al., 1987; Wellems et al., 1990), as well as evaluations of gametocytocidal drugs (Lucantoni et al., 2017) and transmission-blocking vaccine candidates (Goncalves and Hunziker, 2016).

Of the other human malaria parasites, *Plasmodium vivax* and *Plasmodium knowlesi* are also responsible for large public health burdens of illness, predominantly outside of Africa. Despite the closer phylogenetic relationship of these two species than of either to *P. falciparum* (Loy et al., 2017), only *P. knowlesi* has been adapted to continuous culture *in vitro*, in rhesus (Kocken et al., 2002) and in human RBC (Lim et al., 2013; Moon et al.,

2013). Cultivability of these parasites helps to support research on *P. knowlesi* malaria and control of the morbidity and mortality it causes in Southeast Asia (Barber et al., 2017; Yusof et al., 2014). Use of theses parasites may also serve for biological and genetic models of *P. vivax* and the other species that are refractory to continuous cultivation. However, studies of *P. knowlesi* in culture have been limited to the asexual cycle in RBC, as (unlike for *P. falciparum*) no production of sexual stage gametocytes has been reported from a cultivated line (Moon et al., 2013; Zeeman et al., 2013).

P. falciparum gametocytes typically require a development time of 10–12 days (Jeffery and Eyles, 1955) to their mature falciform shape (from which the species takes its name); they then remain infectious for several days (Smalley and Sinden, 1977). In contrast, *P. knowlesi* gametocytes are thought to mature in approximately 1.5 days to large, round forms that fill and enlarge the host RBC; these forms are then infectious for only a matter of hours (Hawking et al., 1968). *P. knowlesi in vivo* infections have a characteristic synchronous 24 h cycle, that includes noontime rupture of schizonts and merozoite invasion of RBC (Hawking et al., 1968). Gametocyte infectivity may thus be periodic, a feature that would maximize transmission efficiency to coincide with the blood-feeding of mosquitoes at night (Hawking et al., 1968), although this has been debated (Mideo et al., 2013). Other human and non-human primate *Plasmodium* species, such as *P. vivax* and *P. cynomolgi*, demonstrate similar rapid, periodic production of mature, infectious gametocytes (Gautret and Motard, 1999; Hawking et al., 1966; Yang, 1996; Yang et al., 1984), in distinction to *P. falciparum* and closely-related *Plasmodia* of the *Laverania* subgenus (Gautret and Motard, 1999).

In our studies, standard “crash” methods that induce gametocytes from *P. falciparum* cultures have failed to increase gamete production from a culture-adapted *P. knowlesi* line derived from the H strain. Here, we show that highly-synchronized *in vitro* cultures of these parasites produced mosquito infections despite low- or even sub-microscopic densities of gametocytes. Synchronized *P. knowlesi* yielded highest infectivity to *An. dirus* mosquitoes 31 – 41 h after purified schizonts ruptured and infected rhesus RBC in freshly established cultures. However, the results varied and in some cases yielded few or no mosquito infections. Gametocytes occurred at low-prevalence and were difficult to observe when mosquito infections developed; transcription from known orthologs of *P. falciparum* gametocyte-specific markers showed no correlation with infectivity of the *P. knowlesi* cultures to mosquitoes.

2. Materials and Methods

2.1 Ethical statement

All nonhuman primates were obtained from National Institutes of Health (NIH) approved sources. All care and use were in accordance with the NIH Animal Research Advisory Committee (NIH ARAC) Guidelines, under protocols approved by the National Institute of Allergy and Infectious Diseases (NIAID) Animal Care and Use Committee and in compliance with the Animal Welfare Act and the Guide for the care and Use of Laboratory Animals.

2.2 Rhesus macaque infections

Four rhesus macaques (*Macaca mulatta*) bred in the U.S. from Indian stock were used in these studies: rhesus DB5KA, male, splenectomized, 12 years old, malaria naïve, 11.92 kg; rhesus FZ8, female, 10 years old, malaria naïve, 7.18 kg; rhesus MCF, female, 4 years old, malaria naïve, 8.26 kg; DB2L, male, splenectomized, 11 years old, malaria naïve, 11.44 kg. Rhesus were anesthetized with ketamine (10 mg/kg body weight) via intramuscular (i.m.) injection prior to inoculations or venipuncture. Animals were inoculated with thawed parasitized RBC (pRBC) or sporozoites from cryopreservates of previous *P. knowlesi* H strain infections in rhesus (cryopreservates kindly provided by Dr. Patrick Duffy, Laboratory of Malaria Immunology and Vaccinology, NIAID Division of Intramural Research). The cryopreserved pRBC were thawed and washed in sterile incomplete RPMI (iRPMI, which is RPMI-1640 medium supplemented with 25 mM Hepes, 10 mg/L hypoxanthine, and 25 mM $NaHCO_3$ (KD Medical, Columbia, MD, USA)) and delivered via intravenous (i.v.) injection to one animal (DB5KA). Cryopreserved sporozoites were thawed at 37°C for 30 s and resuspended in phosphate buffered saline (PBS 1×: 10 mM PO_4^{3-}, 137 mM NaCl, 2.7 mM KCl, pH 7.4) supplemented with 0.6% human serum (Interstate Bloodbank). Approximately 16,500 sporozoites were administered to each of three rhesus (DB2L, FZ8, and MCF) via i.v. injection. Parasitemias of infected animals were monitored daily by microscopy using 20% Giemsa-stained thin films, and up to three times daily after reaching 0.1%. Total parasitemias were obtained after counting an estimated 10,000 RBC. Rhesus infection studies where concluded according to animal protocol when parasitemias of ~5% were detected.

2.3 P. knowlesi *cultivation in vitro*

Uninfected rhesus blood was collected by venipuncture into sodium heparin vacutainers (BD, Franklin Lakes, NJ, USA) and centrifuged at 800 × g for 3 min. Plasma was removed and RBC were washed once and resuspended in iRPMI at 50% hematocrit, and stored at 4ºC for up to two weeks. *P. knowlesi* H strain-infected pRBC were collected from infected rhesus into sodium heparin vacutainers (BD, Franklin Lakes, NJ, USA). After plasma removal by centrifugation, the pRBC were combined with uninfected rhesus RBC in cultures with complete RPMI (cRPMI, which is iRPMI supplemented with 10 mg/L gentamicin, and 1% Albumax II (Life Technologies, Carlsbad, CA, USA)) at 5% hematocrit and 37°C under a 90% N_2, 5% CO_2, and 5% O_2 gas mixture, with media changes once daily, or twice daily if parasitemia was ≥ 4%. Cultures were monitored by microscopy using Giemsa-stained thin films, and maintained at 0.5–10% parasitemia. Total parasitemias and parasite stage distributions were recorded from counts of 5,000 RBC. Cultures were transferred to iRPMI supplemented with 10% (v/v) pooled sera from 2 – 6 animals, for a minimum of three cycles prior to membrane-feeding assays. The three different pools of sera used for this purpose are listed in Table S1.

2.4 Crash method for induction of gametocytes

Attempts to induce *P. knowlesi* gametocytogenesis by culture crash were based on a protocol established for *P. falciparum* (Saliba and Jacobs-Lorena, 2013). Mixed stage PkH/FZ8 cultures were diluted to 0.2% hematocrit with rhesus RBC in iRPMI supplemented with

10% (v/v) rhesus pooled sera. A 75% volume of the overlying medium was removed and replaced every 24 h, but with no addition of RBC, resulting in a small culture volume increase (and corresponding hematocrit reduction) of 1.5-fold after two cycles. Parasitemia was monitored by microscopy using Giemsa-stained thin films to estimate the time at which the asexual parasite population crashed. Cultures were harvested at two-time points following crash of asexual parasites (31 and 36 h) for experimental infection of mosquitoes in membrane-feeding assays.

2.5 Schizont purification for parasite synchronization

A Percoll step-gradient was used to purify *P. knowlesi* schizonts based on a method originally developed for *P. falciparum* (Rivadeneira et al., 1983; Wahlgren et al., 1983). PkH/FZ8 cultures (60 mL) containing >5% asynchronous parasites were pelleted by centrifugation at 800 × *g* for 3 min, and the collected RBC were resuspended into iRPMI to a final 10% hematocrit. This mixture was gently placed onto a 65/35% Percoll (GE Healthcare Life Sciences, Pittsburgh, PA, USA) step-gradient, and centrifuged at 1500 × *g* for 15 min with no brake. The inter-phase band between the 65 and 35% Percoll bands, containing late trophozoites/schizonts, was collected and washed twice with 5 mL of iRPMI. Purified schizonts were confirmed by microscopy of thin film smears. The purified schizonts were returned to culture in iRPMI supplemented with 10% (v/v) rhesus pooled sera, and parasites were harvested at the five-time points (24, 31, 36, 41, and 48 h) for the membrane-feeding assays with mosquitoes.

2.6 *Infection of mosquitoes with* P. knowlesi *by membrane-feeding*

An. dirus (X strain) mosquitoes were reared under standard laboratory conditions on a 12 h light-dark cycle at 27°C and 80% humidity (Sattabongkot et al., 2003). Female (5–7 d) mosquitoes were infected by membrane-feeding (Graves, 1980), with *P. knowlesi* cultures. RBC from cultures were pelleted and resuspended to 50% hematocrit in rhesus serum. Blood was delivered directly into glass, water-jacketed membrane feeders warmed to 37°C, and mosquitoes were allowed to feed through Parafilm M (Bemis Inc., Oshkosh, WI, USA) membranes for up to one hour. Unfed mosquitoes were removed. For oocyst assessments, midguts were dissected from mosquitoes seven days after feeding, stained with 0.1% (w/v) mercurochrome in water, and oocysts enumerated by light microscopy. Salivary glands were dissected and pooled from mosquitoes fed at each of three-time points (31, 36, and 41 h post-synchronization) 14 d after feeding. Salivary gland pairs were homogenized with a pestle (Argos Technologies, Vernon Hills, IL, USA) in a microcentrifuge tube to release sporozoites. Following centrifugation at 6,000 x g for 3 min, the supernatant was collected and sporozoites were counted using a hemocytometer.

2.7 Quantitative real-time PCR analysis

Total RNA was isolated from cultures harvested at five-time points following Percoll synchronization (24, 31, 36, 41, and 48 h) using TRIzol (Thermo Fisher Scientific); residual genomic DNA (gDNA) was removed by TURBO DNase treatment (Thermo Fisher Scientific). cDNA was synthesized using ProtoScript II® First Strand cDNA Synthesis Kit (New England BioLabs). Gene expression analysis was measured by SYBR green qRT-PCR. Primers for qRT-PCR were designed against three known gametocyte markers and one

housekeeping gene (Table S2). Reactions contained 12.5 μL Rotorgene SYBR Green PCR Master Mix (Qiagen), 2 μL (0.3 μM final concentration) each of forward and reverse primers, 2 μL of cDNA and 6.5 μL nuclease free H_20 for a final reaction volume of 25 μL. Reactions were carried out in triplicate using a Roche LightCycler® 480 instrument with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, 68 °C for 30 s and a final extension at 68 °C for 10 min.

The standard curve method was used for absolute quantification of gene expression. Synthetic gene blocks containing the amplicon sequence were used as standards (Table S3). Standard curves were generated by performing 10-fold serial dilutions starting at 7×10^8 copies/μL. For each standard curve, primer efficiency and linear range were calculated (Dataset S1). Standard curves were included in each qRT-PCR run to control for run-to-run variability in amplification efficiency. Data analysis was performed using Roche LightCycler® 480 Software release 1.5.1.62. The Fit Points method was used for absolute quantification. Background fluorescence noise bands were set using the software default setting of 12-fold the standard deviation of the background signal. Unknown sample concentrations were extrapolated by the software using the in-run standard curve. Total copy number was converted to copies/μL by normalizing to the volume of culture from which RNA was extracted. Expression of gametocyte specific markers was further normalized to the transcription of housekeeping gene, *pk serine tRNA ligase* (PKNH_1435500), which serves as a benchmark for gene transcript levels in *Plasmodium* species (Ben Mamoun et al., 2001).

3. Results

3.1 *Culture adapted* P. knowlesi line *retains the ability to form infectious gametocytes*

Standard *in vitro* cultivation conditions (Kocken et al., 2002) were used to adapt a line of the *P. knowlesi* H strain (Chin et al., 1965) that completes the life cycle through infected rhesus (*Macaca mulatta*) and mosquitoes (*An. dirus*) (Murphy et al., 2014). Inoculation of four rhesus with cryopreserved sporozoites or pRBC produced synchronous blood-stage infections with parasitemias increasing up to 15-fold/day (Fig. 1A; Fig. S1). Gametocyte densities of 0.01 – 0.1% were observed along with these parasitemias (Fig. 1B). Blood samples with parasitemias ≥ 1.0% were collected from each infected rhesus to initiate *in vitro* cultures. These cultures were treated as four individual parasite lines (PkH/DB5KA, PkH/DB2L, PkH/FZ8, PkH/MCF), which were successfully cultivated and maintained in rhesus RBC after approximately three weeks of adaptation, in agreement with previous report of *P. knowlesi in vitro* cultivation (Kocken et al., 2002). Based on the counts of *in vivo* gametocytemia and the fact that the PkH/FZ8 infection was initiated from the sporozoites of a recent mosquito infection, we selected this line for focused *in vitro* gametocytogenesis studies. The recovered PkH/FZ8 parasites were maintained in culture at ≤ 6% parasitemia with increases of 2 – 5-fold/day *in vitro* (Fig. 1C); gametocytes under these conditions were not always found, even at higher parasitemias, but were irregularly observed at rates up to 0.05% (Fig. 1D).

To test the infectivity of PkH/FZ8 gametocytes from *in vitro* culture, mixed stage parasites were maintained in media supplemented with 10% rhesus serum and harvested at 5 – 7%

parasitemia for mosquito membrane-feeding assays. Results verified infectivity to *An. dirus*, although the proportion of mosquitoes infected (prevalence: 0.07 – 0.10) and intensity of infections (mean oocysts/midgut: 0.06 – 0.17) were low (Table 1). We next attempted gametocytogenesis by adaptation of a strategy that induces *P. falciparum* gametocytes, allowing the parasitemia to reach high levels and then "crash" in a culture that receives medium changes but no fresh RBC (Saliba and Jacobs-Lorena, 2013). In experiments with the PkH/FZ8 line, culture parasitemias crashed after reaching peaks of 5 – 12% in a period of three or four asexual cycles (Fig. 1E); however, greater numbers of gametocytes were not observed in the crashed than in the routine cultures (Fig. 1F). To test for infectivity of *P. knowlesi* gametocytes, *An. dirus* mosquitoes were membrane fed with RBC from the PkH/FZ8 cultures at 31 and 36 h after crash of the asexual parasitemias. Results showed very few infections of the mosquitoes 7 d after feedings from the cultures: 0.07 – 0.17 and 0.09 – 0.2 mean oocysts per midgut (Table 1).

3.2 *Synchronization of* P. knowlesi *PkH/FZ8 parasites enhance infectivity of P. knowlesi to An. dirus mosquitoes*

We next sought to induce *P. knowlesi* gametocytes *in vitro* by mimicking the synchrony of *in vivo* monkey parasitemias, which may enhance infectivity to mosquitoes during blood feeding hours (Murphy et al., 2014). Mixed stage cultures of PkH/FZ8 were grown to ~5% parasitemia in incomplete RPMI medium (iRPMI) supplemented with 10% naïve rhesus serum. Parasite stages were separated by 65/35% Percoll density gradient centrifugation adapted from a previously described method for *P. falciparum* (Rivadeneira et al., 1983; Wahlgren et al., 1983). Developing and late stage schizonts were collected from the lower interphase (19 – 24 h forms; Fig. S2) and returned to culture at 2% hematocrit in fresh rhesus RBC. The schizont-infected RBC were monitored hourly, and the time at which 50% had burst was designated as the 0 h reference for subsequent time points. Once merozoite egress and invasion of RBC was completed, the parasitemia was adjusted with additional rhesus RBC and culture medium to ~1% rings. Synchrony of stage distributions was found to hold for ≥ 48 h in the cultures (Fig 2A–C, Fig. 3, Fig. S3), but gametocytes were difficult to observe by microscopy (Fig. 2D, Fig. 3). In three of four *in vitro* infections, gametocytes remained at low or unobservable densities, were only detected as females, and showed no clear peak in the time period (Fig. 2D). Efforts were made to check cultures for exflagellation of male gametes at the time of each mosquito membrane feed, but these were without success because of the very rare observations of male gametocytes.

To test infectivity of the synchronized PkH/FZ8 cultures, mosquito membrane-feeding assays were performed at 24, 31, 36, 41, and 48 h after the merozoite invasion from purified schizonts. In six independent experiments, oocysts were readily observed in *An. dirus* mosquitoes 7 d after being fed blood from cultures at the 31 – 41 h timepoints (Fig. 4 A–F). Counts peaked around 36 h, with few or no oocysts detected in mosquitoes fed on cultures 24 or 48 h after synchronization (Table S4; mean 36 h counts 0.05 – 9.07). However, considerable variability of infectivity was observed in six independent experiments, with low counts in three experiments (Fig. 4A, C, E). Among factors that may have affected gametocyte productivity and oocyst counts, we note possible influences of delayed growth kinetics, particularly during early development of ring stages, which may have contributed to

the suboptimal infectivity observed in two experiments (Fig. 2A); other possible factors include RBC sourced from different rhesus and variations among the collected sera (Table S1). Indeed, studies with *P. falciparum* have shown that RBC age can influence gametocyte development (Trager and Gill, 1992; Trager et al., 1999), and sera samples are known to differ in their ability to support gametocyte growth (Delves et al., 2016). Fitness and vector competence can shift with seemingly small changes in the laboratory practices of rearing mosquitoes (Gilles et al., 2011; Mwangangi et al., 2007). Finally, parasites in long-term asexual culture can lose their ability to produce gametocytes, *e.g.* as documented with strains of *P. falciparum* (Alano et al., 1995; Furuya et al., 2005); however, this possibility seems unlikely for the *P. knowlesi* in our experiments since oocyst production was successful from parasites in continuous culture for 48 – 200 days (Table S1).

Mosquitoes from two experiments, 2 and 4 (Fig. 4B, D), were maintained and checked for the presence of sporozoites 14 d post-blood-feeding. Maximum counts of 17,160 ± 4,575 (mean ± SEM, n=21) sporozoites per mosquito were obtained from infections with parasites 36 h after synchronization, whereas 4,494 ± 1,906 (n=20) and 7,275 ± 5,058 (n=22) sporozoites per mosquito were obtained from infections with parasites 31 or 41 h following synchronization, respectively (Table S5).

3.3 *Transcription of* P. knowlesi *orthologs of* P. falciparum *gametocyte markers*

In further assessments of *P. knowlesi* gametocyte development and maturation, we identified orthologs of *P. falciparum* genes expressed in gametocytes and quantified their transcription by RT-qPCR. These experiments included the *pks16* ortholog *of pfs16* that is expressed in all sexual forms of *P. falciparum*, including sexually committed ring stages (Bruce et al., 1994), and the *pks25* and *pks47* orthologs of the *P. falciparum* mature female gametocyte markers *pfs25* and *pfs47* (Schneider et al., 2015; van Schaijk et al., 2006). Normalization for parasite levels in these assessments was calculated relative to transcription from the single-copy constitutive gene for *P. knowlesi* serine tRNA ligase (PKNH_1435500), which exhibits an expression profile similar to the curve of parasitemia in culture (compare Fig. 5A and S3). Consistent with its homology to *pfs16* expressed in *P. falciparum* cultures (Lanfrancotti et al., 2007), *pks16* transcripts were readily detected in the *P. knowlesi* cultures. Transcription of *pks16* was reduced at the 36 h time-point in five of the six experiments, after which average transcription rebounded (Fig. 5B); however, these reductions were not good predictors of infection across the individual experiments. Transcription of *pks25* varied considerably among the six experiments early on and then was more consistent at the 30 – 48 h time points, but with no clear peaks that could be related to gametocyte maturation or infectivity (Fig. 5C). Likewise, consistent transcription was observed from *pks47* except for a low level in one experiment at the 36 h time point (Fig. 5D). Unlike *pfs47*, which is a single copy gene, three copies of *pks47* are present in the *P. knowlesi* H strain genome, of which two present complete open reading frames (ORFs) and the third copy is truncated (Pain et al., 2008). Our primers were designed to amplify and detect transcripts from any of the three copies.

4. Discussion

Asexually propagating *Plasmodium* parasites transmit from the vertebrate bloodstream by committing a fraction of their number to sexual stage gametocytes for mosquito infections. Methods for *Plasmodium* gametocyte cultivation and mosquito infection are therefore valuable laboratory tools for studies of malaria transmission and the discovery of methods to interrupt it. Production of gametocytes from *in vitro* cultures has previously been established only for *P. falciparum*. Here, we have shown that synchronization by density centrifugation can yield gametocytes and mosquito infections from a *P. knowlesi* line cultivated continuously in rhesus RBC.

Early events of *Plasmodium* sexual differentiation and gametocyte development are initiated by the apicomplexan-specific transcription factor ApiAP2-G (Kafsack et al., 2014; Sinha et al., 2014), which is epigenetically regulated (Brancucci et al., 2014; Coleman et al., 2014). Epigenetic regulation is thought to establish levels of gametocytogenesis that can fluctuate in response to environmental stimuli or stressors (Bechtsi and Waters, 2017) including host immune pressure (Smalley and Brown, 1981), hormones (Escobedo et al., 2005), high parasitemia (Chaubey et al., 2014), hemolysis (Schneweis et al., 1991), and anti-malarial drugs (Butcher, 1997). With *P. falciparum* cultures, experimental conditions for gametocyte induction are achieved by allowing asexual populations to expand in the culture for a period of 14 – 19 days, with daily medium changes but without any addition of fresh RBC (“crash” method) (Ifediba and Vanderberg, 1981; Saliba and Jacobs-Lorena, 2013). This method can be modified by altering culture volume to decrease or increase hematocrit (Ruecker et al., 2014; Tao et al., 2014), or by using parasite-conditioned medium (Dyer and Day, 2003; Ponnudurai et al., 1982). In our experiments with cultivated *P. knowlesi* parasites, the failure of the “crash” method to improve gametocytogenesis or increase mosquito infections above the low levels in routinely-maintained cultures was unexpected. Potential explanations include insufficient stimuli from the environment of *P. knowlesi* culture without fresh RBC additions, or that *P. knowlesi* is inherently less responsive than *P. falciparum* to parasitemia crash effects.

In contrast to poor gametocytogenesis and mosquito infectivity from crash conditions, mosquito infections were successful with synchronized *P. knowlesi* populations grown from Percoll gradient-purified schizonts. Although gametocytes were observed only at low density in these populations, results from the successful membrane-feeding assays demonstrated the presence of mature, infective *P. knowlesi* gametocytes 31 – 41 h after the first invasion of synchronized merozoites. This short-term, periodic, infectivity of *P. knowlesi* gametocytes has been previously reported from *in vivo* infections (Hawking et al., 1968). Transmission of *P. knowlesi* gametocytes from the synchronized cultures, as quantified by numbers of oocysts and sporozoites per mosquito, compares to transmissions achieved with 0.1–0.3% densities of *P. falciparum* gametocytes fed to laboratory mosquitoes (Miura et al., 2013; Saliba and Jacobs-Lorena, 2013). However, because of their low densities, their morphological similarity to trophozoites, and the lack of availability distinguishing molecular markers, we were unable to accurately quantify *P. knowlesi* gametocytogenesis or track the development of mature gametocytes. Also, the complete absence of observable exflagellation events excluded the use of these as a proxy for

gametocyte maturation or infectivity. It thus remains unclear if synchronization may coordinate signaling of a population of gametocytes above a density threshold necessary for effective mosquito infection, or if the method otherwise induces gametocytogenesis by stress of experimental manipulation, *e.g.* exposure to Percoll which can affect viability under some circumstances (Spadafora et al., 2011). New markers and methods for gametocyte detection will support research on these and other questions in the biology of *P. knowlesi* transmission, including studies of mosquito infection for approaches to malaria control.

Gametocyte induction and mosquito infections from *in vitro* cultures offer new opportunities for investigations of the complete *P. knowlesi* life cycle in the laboratory. Mosquito infections may also be possible with *P. knowlesi* parasites adapted to culture in human RBC, thereby avoiding the expense and difficulty of using rhesus RBC. With modern molecular methods including genetic manipulation (Kocken et al., 2002; Moon et al., 2013; Moon et al., 2016; Moraes Barros et al., 2017), research will advance our understanding of the transmission of *P. knowlesi, P. vivax* and related *Plasmodium* species, with potential applications for assays and interventions in malaria control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the Intramural Research Program of the Division of Intramural Research of the National Institute of Allergy and Infectious Disease, National Institutes of Health (NIH). J.S.A. was supported by a grant from the Bill and Melinda Gates Foundation (OPP#1023643) awarded to J.H.A. We thank Andre Laughinghouse and Kevin Lee for insectary support, and MarvinT. Thomas, Theresa Engels, Milton Herrera, Kelly Dicken, and Billetta Lewis at the Division of Veterinary Resources, NIH, for technical assistance with non-human primates. We are thankful to Patrick Duffy and Salomon Conteh for providing the parasite line, and Jingyang Chen, Carolina Barillas-Murray, and Caeul Lim for helpful discussions.

References

Alano P, Roca L, Smith D, Read D, Carter R, Day K, 1995 Plasmodium falciparum: parasites defective in early stages of gametocytogenesis. Exp Parasitol 81, 227–235. [PubMed: 7556565]

Barber BE, Grigg MJ, William T, Yeo TW, Anstey NM, 2017 The Treatment of Plasmodium knowlesi Malaria. Trends Parasitol 33, 242–253. [PubMed: 27707609]

Bechtsi DP, Waters AP, 2017 Genomics and epigenetics of sexual commitment in Plasmodium. Int J Parasitol 47, 425–434. [PubMed: 28455236]

Ben Mamoun C, Gluzman IY, Hott C, MacMillan SK, Amarakone AS, Anderson DL, Carlton JM, Dame JB, Chakrabarti D, Martin RK, Brownstein BH, Goldberg DE, 2001 Co-ordinated programme of gene expression during asexual intraerythrocytic development of the human malaria parasite Plasmodium falciparum revealed by microarray analysis. Mol Microbiol 39, 26–36. [PubMed: 11123685]

Brancucci NMB, Bertschi NL, Zhu L, Niederwieser I, Chin WH, Wampfler R, Freymond C, Rottmann M, Felger I, Bozdech Z, Voss TS, 2014 Heterochromatin protein 1 secures survival and transmission of malaria parasites. Cell Host Microbe 16, 165–176. [PubMed: 25121746]

Bruce MC, Carter RN, Nakamura K, Aikawa M, Carter R, 1994 Cellular location and temporal expression of the Plasmodium falciparum sexual stage antigen Pfs16. Mol Biochem Parasitol 65, 11–22. [PubMed: 7935618]

Butcher GA, 1997 Antimalarial drugs and the mosquito transmission of Plasmodium. Int J Parasitol 27, 975–987. [PubMed: 9363480]

Chaubey S, Grover M, Tatu U, 2014 Endoplasmic reticulum stress triggers gametocytogenesis in the malaria parasite. J Biol Chem 289, 16662–16674. [PubMed: 24755215]

Chin W, Contacos PG, Coatney GR, Kimball HR, 1965 A naturally acquired quotidian-type malaria in man transferable to monkeys. Science 149, 865. [PubMed: 14332847]

Coleman BI, Skillman KM, Jiang RHY, Childs LM, Altenhofen LM, Ganter M, Leung Y, Goldowitz I, Kafsack BFC, Marti M, Llinas M, Buckee CO, Duraisingh MT, 2014 A Plasmodium falciparum histone deacetylase regulates antigenic variation and gametocyte conversion. Cell Host Microbe 16, 177–186. [PubMed: 25121747]

Delves MJ, Straschil U, Ruecker A, Miguel-Blanco C, Marques S, Dufour AC, Baum J, Sinden RE, 2016 Routine in vitro culture of P. falciparum gametocytes to evaluate novel transmission-blocking interventions. Nat Protoc 11, 1668–1680. [PubMed: 27560172]

Dyer M, Day KP, 2003 Regulation of the rate of asexual growth and commitment to sexual development by diffusible factors from in vitro cultures of Plasmodium falciparum. Am J Trop Med Hyg 68, 403–409. [PubMed: 12875287]

Escobedo G, Roberts CW, Carrero JC, Morales-Montor J, 2005 Parasite regulation by host hormones: an old mechanism of host exploitation? Trends Parasitol 21, 588–593. [PubMed: 16236553]

Furuya T, Mu J, Hayton K, Liu A, Duan J, Nkrumah L, Joy DA, Fidock DA, Fujioka H, Vaidya AB, Wellems TE, Su XZ, 2005 Disruption of a Plasmodium falciparum gene linked to male sexual development causes early arrest in gametocytogenesis. Proc Natl Acad Sci U S A 102, 16813–16818. [PubMed: 16275909]

Gautret P, Motard A, 1999 Periodic infectivity of Plasmodium gametocytes to the vector. A review. Parasite 6, 103–111. [PubMed: 10416184]

Gilles JR, Lees RS, Soliban SM, Benedict MQ, 2011 Density-dependent effects in experimental larval populations of Anopheles arabiensis (Diptera: Culicidae) can be negative, neutral, or overcompensatory depending on density and diet levels. J Med Entomol 48, 296–304. [PubMed: 21485365]

Goncalves D, Hunziker P, 2016 Transmission-blocking strategies: the roadmap from laboratory bench to the community. Malar J 15, 95. [PubMed: 26888537]

Graves PM, 1980 Studies on the use of a membrane feeding technique for infecting Anopheles gambiae with Plasmodium falciparum. Trans R Soc Trop Med Hyg 74, 738–742. [PubMed: 7010696]

Hawking F, Worms MJ, Gammage K, 1968 Host temperature and control of 24-hour and 48-hour cycles in malaria parasites. Lancet 1, 506–509. [PubMed: 4170834]

Hawking F, Worms MJ, Gammage K, Goddard PA, 1966 Biological purpose of blood-cycle of malaria parasite Plasmodium Cynomolgi. Lancet 2, 422-&.

Haynes JD, Diggs CL, Hines FA, Desjardins RE, 1976 Culture of human malaria parasites Plasmodium falciparum. Nature 263, 767–769. [PubMed: 825784]

Hayton K, Gaur D, Liu A, Takahashi J, Henschen B, Singh S, Lambert L, Furuya T, Bouttenot R, Doll M, Nawaz F, Mu J, Jiang L, Miller LH, Wellems TE, 2008 Erythrocyte binding protein PfRH5 polymorphisms determine species-specific pathways of Plasmodium falciparum invasion. Cell Host Microbe 4, 40–51. [PubMed: 18621009]

Ifediba T, Vanderberg JP, 1981 Complete in vitro maturation of Plasmodium falciparum gametocytes. Nature 294, 364–366. [PubMed: 7031476]

Jeffery GM, Eyles DE, 1955 Infectivity to mosquitoes of Plasmodium falciparum as related to gametocyte density and duration of infection. Am J Trop Med Hyg 4, 781–789. [PubMed: 13259002]

Kafsack BF, Rovira-Graells N, Clark TG, Bancells C, Crowley VM, Campino SG, Williams AE, Drought LG, Kwiatkowski DP, Baker DA, Cortes A, Llinas M, 2014 A transcriptional switch underlies commitment to sexual development in malaria parasites. Nature 507, 248–252. [PubMed: 24572369]

Kocken CH, Ozwara H, van der Wel A, Beetsma AL, Mwenda JM, Thomas AW, 2002 Plasmodium knowlesi provides a rapid in vitro and in vivo transfection system that enables double-crossover gene knockout studies. Infect Immun 70, 655–660. [PubMed: 11796595]

Lanfrancotti A, Bertuccini L, Silvestrini F, Alano P, 2007 Plasmodium falciparum: mRNA co-expression and protein co-localisation of two gene products upregulated in early gametocytes. Exp Parasitol 116, 497–503. [PubMed: 17367781]

Lim C, Hansen E, DeSimone TM, Moreno Y, Junker K, Bei A, Brugnara C, Buckee CO, Duraisingh MT, 2013 Expansion of host cellular niche can drive adaptation of a zoonotic malaria parasite to humans. Nat Commun 4, 1638. [PubMed: 23535659]

Loy DE, Liu W, Li Y, Learn GH, Plenderleith LJ, Sundararaman SA, Sharp PM, Hahn BH, 2017 Out of Africa: origins and evolution of the human malaria parasites Plasmodium falciparum and Plasmodium vivax. Int J Parasitol 47, 87–97. [PubMed: 27381764]

Lucantoni L, Loganathan S, Avery VM, 2017 The need to compare: assessing the level of agreement of three high-throughput assays against Plasmodium falciparum mature gametocytes. Sci Rep 7, 45992. [PubMed: 28378767]

Mantel PY, Hoang AN, Goldowitz I, Potashnikova D, Hamza B, Vorobjev I, Ghiran I, Toner M, Irimia D, Ivanov AR, Barteneva N, Marti M, 2013 Malaria-infected erythrocyte-derived microvesicles mediate cellular communication within the parasite population and with the host immune system. Cell Host Microbe 13, 521–534. [PubMed: 23684304]

Meibalan E, Marti M, 2017 Biology of malaria transmission. Cold Spring Harb Perspect Med 7.

Mideo N, Reece SE, Smith AL, Metcalf CJ, 2013 The Cinderella syndrome: why do malaria-infected cells burst at midnight? Trends Parasitol 29, 10–16. [PubMed: 23253515]

Miura K, Deng B, Tullo G, Diouf A, Moretz SE, Locke E, Morin M, Fay MP, Long CA, 2013 Qualification of standard membrane-feeding assay with Plasmodium falciparum malaria and potential improvements for future assays. PLoS One 8, e57909. [PubMed: 23483940]

Moon RW, Hall J, Rangkuti F, Ho YS, Almond N, Mitchell GH, Pain A, Holder AA, Blackman MJ, 2013 Adaptation of the genetically tractable malaria pathogen Plasmodium knowlesi to continuous culture in human erythrocytes. Proc Natl Acad Sci U S A 110, 531–536. [PubMed: 23267069]

Moon RW, Sharaf H, Hastings CH, Ho YS, Nair MB, Rchiad Z, Knuepfer E, Ramaprasad A, Mohring F, Amir A, Yusuf NA, Hall J, Almond N, Lau YL, Pain A, Blackman MJ, Holder AA, 2016 Normocyte-binding protein required for human erythrocyte invasion by the zoonotic malaria parasite Plasmodium knowlesi. Proc Natl Acad Sci U S A 113, 7231–7236. [PubMed: 27303038]

Moraes Barros RR, Gibson TJ, Kite WA, Sa JM, Wellems TE, 2017 Comparison of two methods for transformation of Plasmodium knowlesi: Direct schizont electroporation and spontaneous plasmid uptake from plasmid-loaded red blood cells. Mol Biochem Parasitol 218, 16–22. [PubMed: 28988930]

Murphy JR, Weiss WR, Fryauff D, Dowler M, Savransky T, Stoyanov C, Muratova O, Lambert L, Orr-Gonzalez S, Zeleski KL, Hinderer J, Fay MP, Joshi G, Gwadz RW, Richie TL, Villasante EF, Richardson JH, Duffy PE, Chen J, 2014 Using infective mosquitoes to challenge monkeys with Plasmodium knowlesi in malaria vaccine studies. Malar J 13, 215. [PubMed: 24893777]

Mwangangi JM, Mbogo CM, Muturi EJ, Nzovua JG, Kabiru EW, Githure JI, Novak RJ, Beier JC, 2007 Influence of biological and physicochemical characteristics of larval habitats on the body size of Anopheles gambiae mosquitoes (Diptera: Culicidae) along the Kenyan coast. J Vector Borne Dis 44, 122–127. [PubMed: 17722866]

Pain A, Bohme U, Berry AE, Mungall K, Finn RD, Jackson AP, Mourier T, Mistry J, Pasini EM, Aslett MA, Balasubrammaniam S, Borgwardt K, Brooks K, Carret C, Carver TJ, Cherevach I, Chillingworth T, Clark TG, Galinski MR, Hall N, Harper D, Harris D, Hauser H, Ivens A, Janssen CS, Keane T, Larke N, Lapp S, Marti M, Moule S, Meyer IM, Ormond D, Peters N, Sanders M, Sanders S, Sargeant TJ, Simmonds M, Smith F, Squares R, Thurston S, Tivey AR, Walker D, White B, Zuiderwijk E, Churcher C, Quail MA, Cowman AF, Turner CM, Rajandream MA, Kocken CH, Thomas AW, Newbold CI, Barrell BG, Berriman M, 2008 The genome of the simian and human malaria parasite Plasmodium knowlesi. Nature 455, 799–803. [PubMed: 18843368]

Ponnudurai T, Meuwissen JH, Leeuwenberg AD, Verhave JP, Lensen AH, 1982 The production of mature gametocytes of Plasmodium falciparum in continuous cultures of different isolates infective to mosquitoes. Trans R Soc Trop Med Hyg 76, 242–250. [PubMed: 7048650]

Regev-Rudzki N, Wilson DW, Carvalho TG, Sisquella X, Coleman BM, Rug M, Bursac D, Angrisano F, Gee M, Hill AF, Baum J, Cowman AF, 2013 Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. Cell 153, 1120–1133. [PubMed: 23683579]

Rivadeneira EM, Wasserman M, Espinal CT, 1983 Separation and concentration of schizonts of Plasmodium falciparum by Percoll gradients. J Protozool 30, 367–370. [PubMed: 6313915]

Ruecker A, Mathias DK, Straschil U, Churcher TS, Dinglasan RR, Leroy D, Sinden RE, Delves MJ, 2014 A male and female gametocyte functional viability assay to identify biologically relevant malaria transmission-blocking drugs. Antimicrob Agents Chemother 58, 7292–7302. [PubMed: 25267664]

Saliba KS, Jacobs-Lorena M, 2013 Production of Plasmodium falciparum gametocytes in vitro. Methods Mol Biol 923, 17–25. [PubMed: 22990768]

Sattabongkot J, Maneechai N, Phunkitchar V, Eikarat N, Khuntirat B, Sirichaisinthop J, Burge R, Coleman RE, 2003 Comparison of artificial membrane feeding with direct skin feeding to estimate the infectiousness of Plasmodium vivax gametocyte carriers to mosquitoes. Am J Trop Med Hyg 69, 529–535. [PubMed: 14695091]

Schneider P, Reece SE, van Schaijk BC, Bousema T, Lanke KH, Meaden CS, Gadalla A, Ranford-Cartwright LC, Babiker HA, 2015 Quantification of female and male Plasmodium falciparum gametocytes by reverse transcriptase quantitative PCR. Mol Biochem Parasitol 199, 29–33. [PubMed: 25827756]

Schneweis S, Maier WA, Seitz HM, 1991 Haemolysis of infected erythrocytes--a trigger for formation of Plasmodium falciparum gametocytes? Parasitol Res 77, 458–460. [PubMed: 1891456]

Sinha A, Hughes KR, Modrzynska KK, Otto TD, Pfander C, Dickens NJ, Religa AA, Bushell E, Graham AL, Cameron R, Kafsack BFC, Williams AE, Llinas M, Berriman M, Billker O, Waters AP, 2014 A cascade of DNA-binding proteins for sexual commitment and development in Plasmodium. Nature 507, 253–257. [PubMed: 24572359]

Smalley ME, Brown J, 1981 Plasmodium falciparum gametocytogenesis stimulated by lymphocytes and serum from infected Gambian children. Trans R Soc Trop Med Hyg 75, 316–317. [PubMed: 7029805]

Smalley ME, Sinden RE, 1977 Plasmodium falciparum gametocytes: their longevity and infectivity. Parasitology 74, 1–8. [PubMed: 320542]

Spadafora C, Gerena L, Kopydlowski KM, 2011 Comparison of the in vitro invasive capabilities of Plasmodium falciparum schizonts isolated by Percoll gradient or using magnetic based separation. Malar J 10, 96. [PubMed: 21501476]

Tao D, Ubaida-Mohien C, Mathias DK, King JG, Pastrana-Mena R, Tripathi A, Goldowitz I, Graham DR, Moss E, Marti M, Dinglasan RR, 2014 Sex-partitioning of the Plasmodium falciparum stage V gametocyte proteome provides insight into falciparum-specific cell biology. Mol Cell Proteomics 13, 2705–2724. [PubMed: 25056935]

Trager W, Gill GS, 1992 Enhanced gametocyte formation in young erythrocytes by Plasmodium falciparum in vitro. J Protozool 39, 429–432. [PubMed: 1640389]

Trager W, Gill GS, Lawrence C, Nagel RL, 1999 Plasmodium falciparum: enhanced gametocyte formation in vitro in reticulocyte-rich blood. Exp Parasitol 91, 115–118. [PubMed: 9990338]

Trager W, Jensen JB, 1976 Human malaria parasites in continuous culture. Science 193, 673–675. [PubMed: 781840]

van Schaijk BC, van Dijk MR, van de Vegte-Bolmer M, van Gemert GJ, van Dooren MW, Eksi S, Roeffen WF, Janse CJ, Waters AP, Sauerwein RW, 2006 Pfs47, paralog of the male fertility factor Pfs48/45, is a female specific surface protein in Plasmodium falciparum. Mol Biochem Parasitol 149, 216–222. [PubMed: 16824624]

Wahlgren M, Berzins K, Perlmann P, Bjorkman A, 1983 Characterization of the humoral immune response in Plasmodium falciparum malaria. I. Estimation of antibodies to P. falciparum or human erythrocytes by means of microELISA. Clin Exp Immunol 54, 127–134. [PubMed: 6352102]

Walliker D, Quakyi IA, Wellems TE, McCutchan TF, Szarfman A, London WT, Corcoran LM, Burkot TR, Carter R, 1987 Genetic analysis of the human malaria parasite Plasmodium falciparum. Science 236, 1661–1666. [PubMed: 3299700]

Wellems TE, Panton LJ, Gluzman IY, do Rosario VE, Gwadz RW, Walker-Jonah A, Krogstad DJ, 1990 Chloroquine resistance not linked to mdr-like genes in a Plasmodium falciparum cross. Nature 345, 253–255. [PubMed: 1970614]

Yang B, 1996 Experimental and field studies on some biological characteristics of Plasmodium vivax isolated from tropical and temperate zones of China. Chin Med J (Engl) 109, 266–271. [PubMed: 8758285]

Yang BL, Wan WJ, Wang WR, Hu HX, Li HX, Wang XA, Li XL, Li CZ, Huang GZ, 1984 [Experimental studies on the periodical viability of gametocytes of Plasmodium vivax]. Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 2, 238–242. [PubMed: 6394172]

Yusof R, Lau YL, Mahmud R, Fong MY, Jelip J, Ngian HU, Mustakim S, Hussin HM, Marzuki N, Mohd Ali M, 2014 High proportion of knowlesi malaria in recent malaria cases in Malaysia. Malar J 13, 168. [PubMed: 24886266]

Zeeman AM, der Wel AV, Kocken CH, 2013 Ex vivo culture of Plasmodium vivax and Plasmodium cynomolgi and in vitro culture of Plasmodium knowlesi blood stages. Methods Mol Biol 923, 35–49. [PubMed: 22990770]

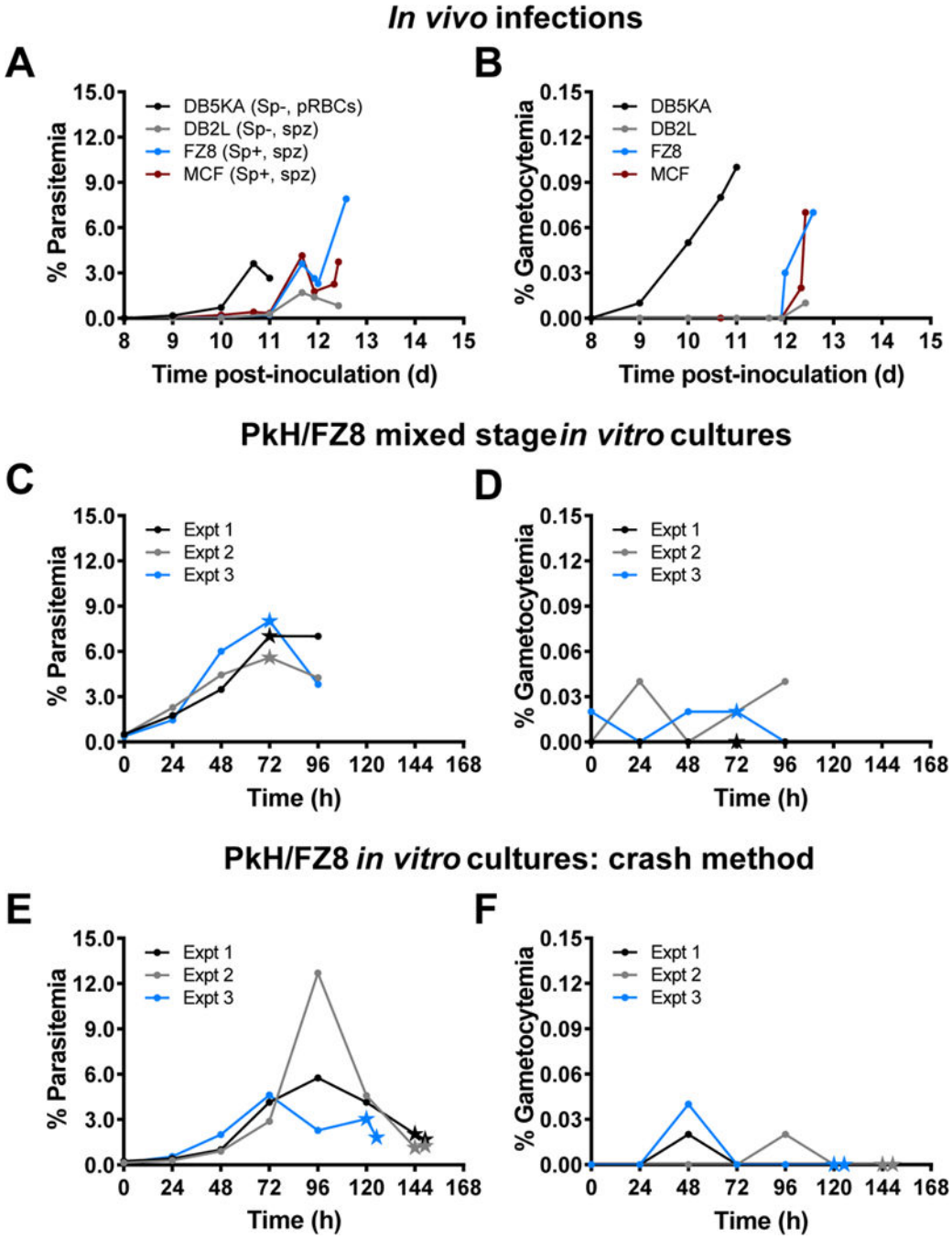


Fig. 1. *P. knowlesi* H parasites produce infectious gametocytes after adaptation to *in vitro* culture.

(*A*) Asexual blood-stage parasitemias developed in splenectomized (Sp-) or spleen intact (Sp +) rhesus from infections with pRBC as well as sporozoites (spz) of the *P. knowlesi* H line. Rhesus DB5KA was inoculated with cryopreserved pRBC and DB2L, FZ8, and MCF were inoculated with 16,500 cryopreserved sporozoites each. (*B*) Gametocytes were detected in all infections and increased with the development of parasitemia. Parasitemias and parasite stages were determined by counting an estimated 10,000 RBC on thin blood films up to three times daily. Experiments were concluded once parasitemias reached 5%, according to

approved animal study protocols. (*C*) Following adaptation to *in vitro* culture, *P. knowlesi* H strain FZ8 (PkH/FZ8) parasitemias increased 2 – 5-fold with each cycle, while (*D*) gametocytes remained at low levels. Slower growth and decreased counts of healthy parasites were observed when the *in vitro* parasitemias rose above 5% after 72 h, consistent with "crash" conditions. (*E*) PkH/FZ8 cultures crashed after 3 cycles without the addition of fresh RBC, but (*F*) this did not increase gametocytemia. Culture parasitemias were determined by counting an estimated 5,000 RBC on thin blood films. Stars in *C-F* denote when mosquito membrane-feeding assays were performed.

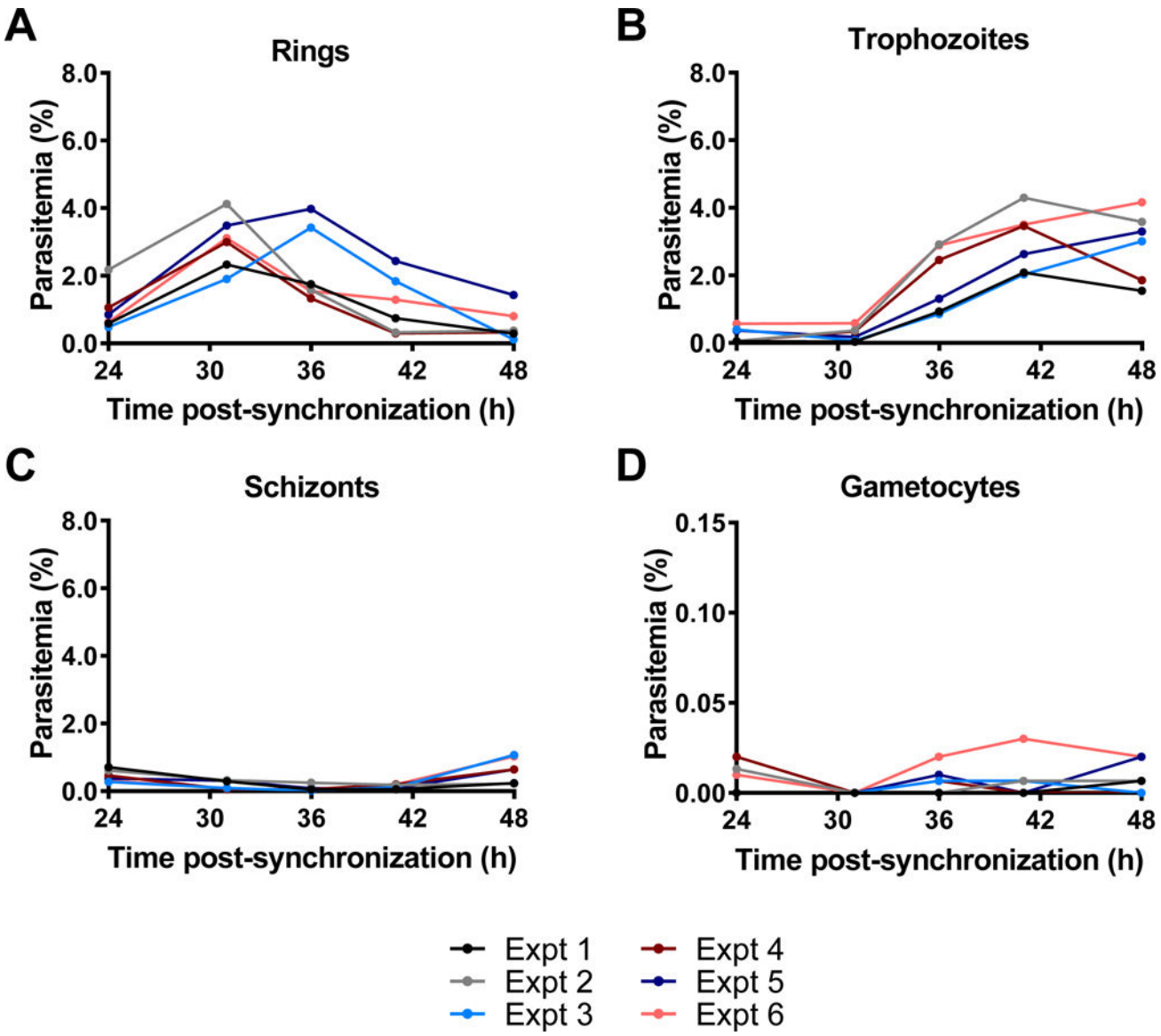


Fig. 2. *P. knowlesi* H parasites exhibit synchrony in cultures seeded with Percoll gradient-purified schizonts.

Ring stages (*A*), trophozoites (*B*), and schizonts (*C*) retain synchrony into the second 24 h cycle of asexual stage growth after introduction of Percoll gradient-purified schizonts into *in vitro* culture with fresh rhesus RBC. Low densities of gametocytes were detected at most time points (*D*). The 0 h reference was set as the point when 50% of schizont-infected RBC had released merozoites for invasion. *P. knowlesi* parasites from *in vitro* cultures were quantified and staged by examination of 5,000 RBC in Giemsa-stained thin blood films every 6 h in the 24 – 48 h period.

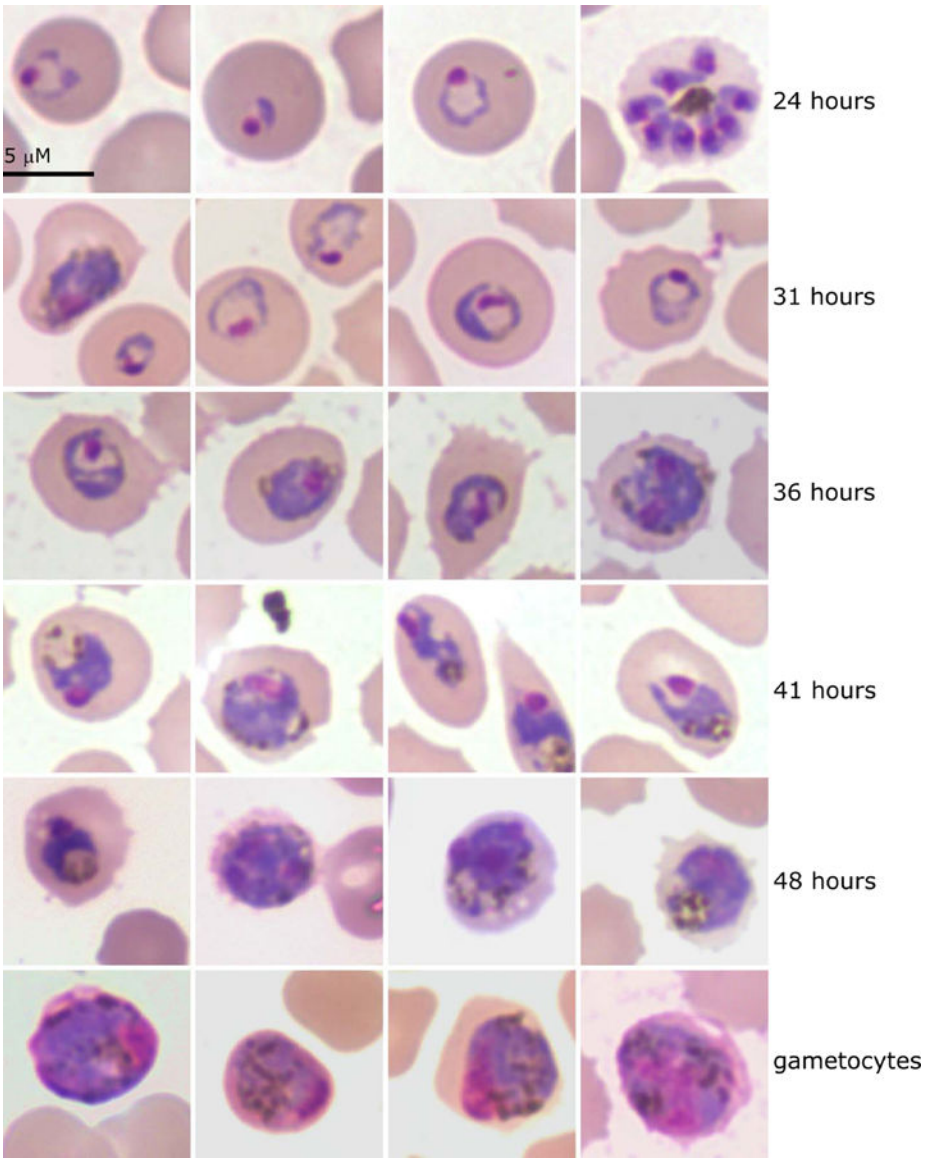


Fig. 3. Images of *P. knowlesi* pRBC following synchronization of *in vitro* cultures.

Images show representative *P. knowlesi* stages at timepoints in synchronized cultures initiated by merozoites released from Percoll gradient-purified schizonts. Starting timepoint of 0 hours was defined by release of merozoites from 50% of the schizonts. Early ring stages and late mature schizonts are evident in the images from Giemsa-stained blood films at 24 h; later stage rings and early trophozoites at 31 hours; more mature trophozoites at 36 h. Developing and mature schizonts reappear at the 41 h and 48 h timepoints. Gametocytes (bottom panels) were detected in low prevalence at all time points.

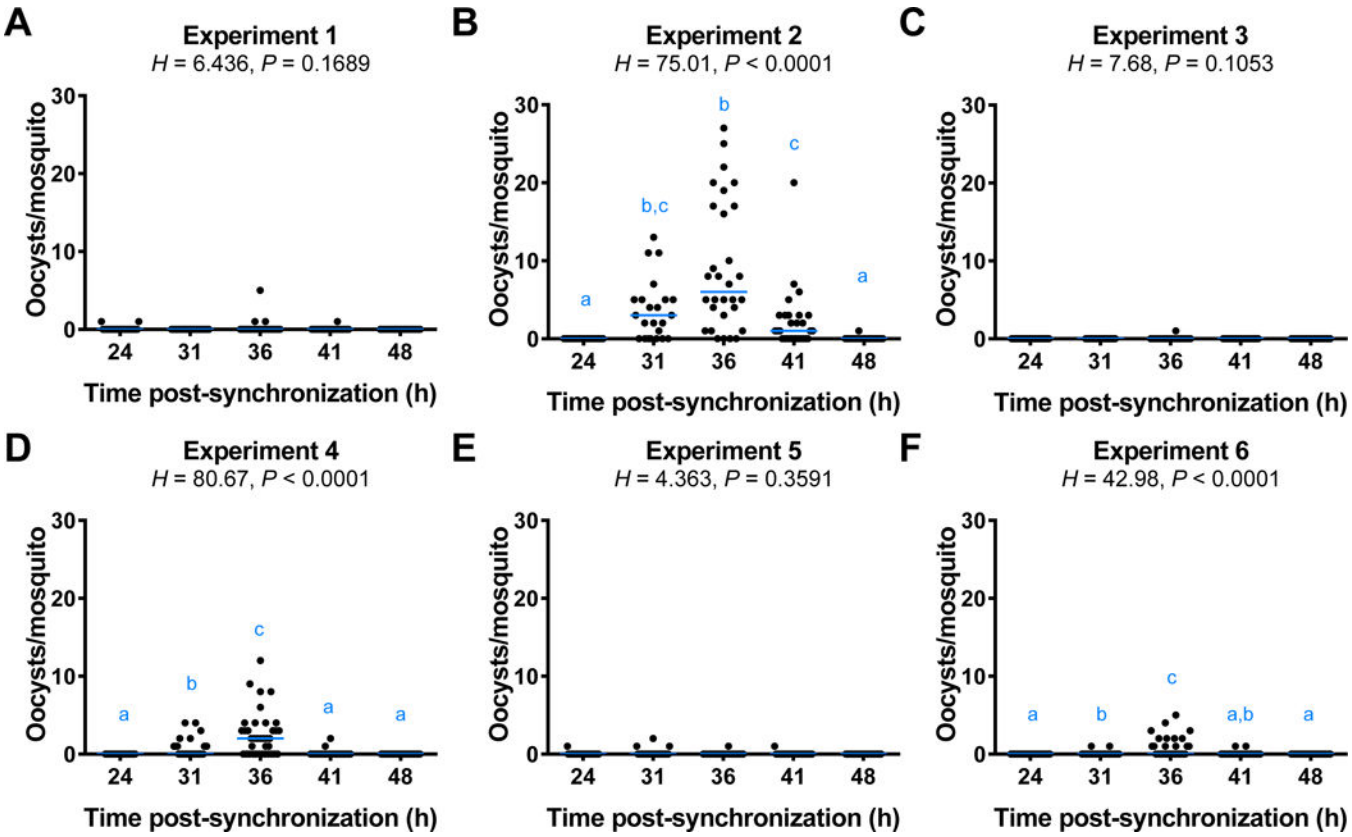


Fig. 4. Synchronized cultures of *P. knowlesi* parasites yield infections of *An. dirus* mosquitos.

Samples of synchronized *P. knowlesi* H parasites were membrane-fed to *An. dirus* mosquitoes at the 24, 31, 36, 41, and 48 h timepoints of six independent experiments (*A*-*F*). Counts of oocysts in the mosquitoes 7 d after bloodfeeding revealed an overall peak of transmission at 36 h. Horizontal bars represent medians. Kruskal-Wallis *H* statistics and *P* values are shown for each experiment. Groups that do not share a letter are significantly different by Dunn's multiple comparison tests.

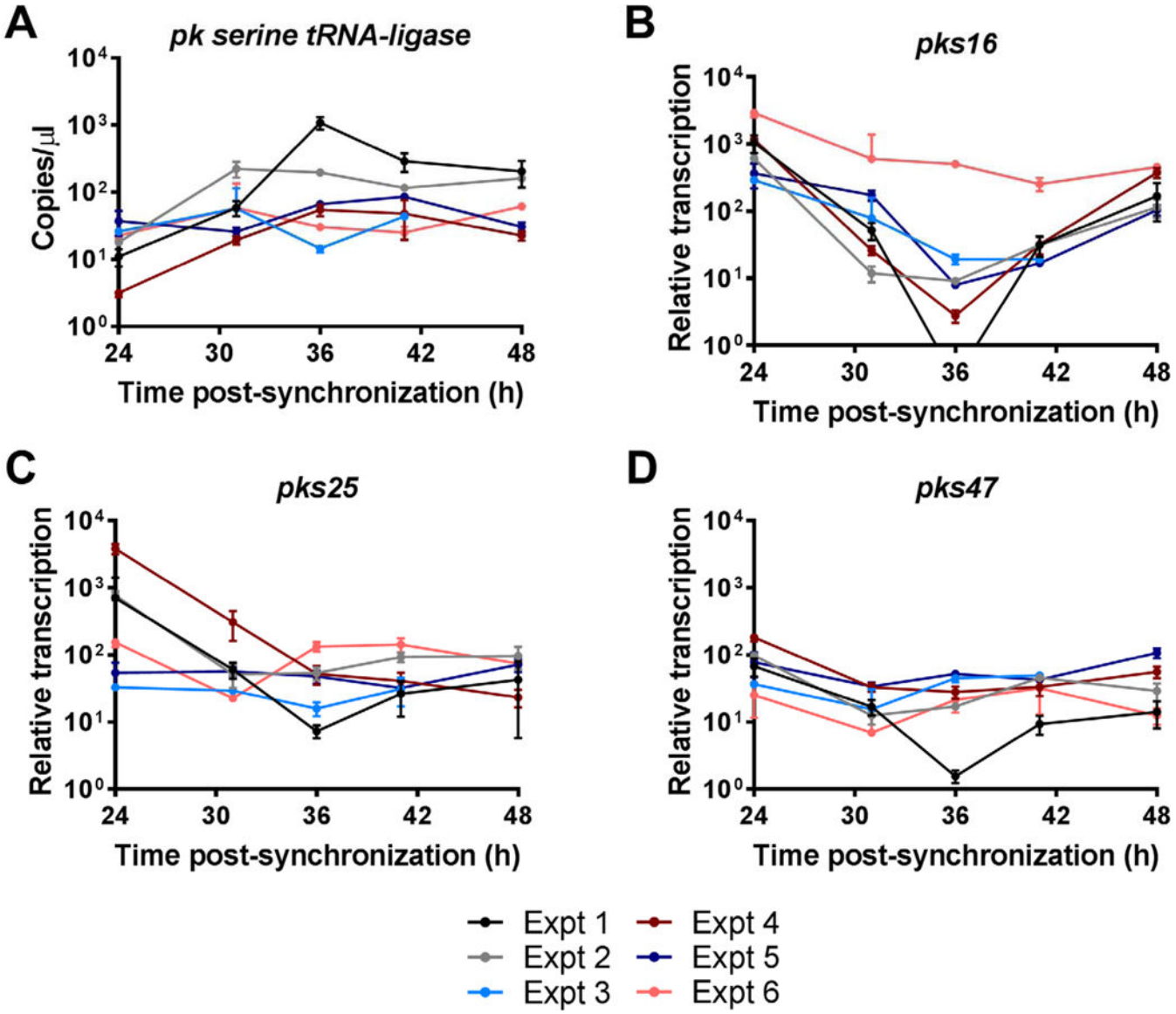


Fig. 5. Mosquito infections are not predicted by *P. knowlesi* transcripts from orthologs of genes expressed in *P. falciparum* gametocytes.

Transcription of putative *P. knowlesi* gametocyte markers *pks16*, *pks25*, and *pks47* was determined relative to transcription from the single copy, constitutive *pk serine tRNA ligase* gene. Results from the RT-qPCR experiments are shown at the 24, 31, 36, 41, and 48 h timepoints of six independent experiments with synchronized cultures of *P. knowlesi* H parasites. (*A*) Plots show the transcription levels from the *pk serine tRNA ligase gene* (copies/μL) used to quantify parasitemia in the six experiments. Increased expression between 24 and 36 h coincided with increased parasitemia observed over this period by microscopy. (*B*) Transcript levels of the *pks16* ortholog of the *P. falciparum pfs16* gametocyte marker are reduced at the 36 h time-point in five of the six experiments, but these reductions did not correlate with *P. knowlesi* oocysts counts as an indicator of mosquito infectivity. (*C, D*) Transcription of the *pks25* and *pks47* orthologs of the *P.*

falciparum mature female gametocyte markers *pfs25* and *pfs47 pks25* likewise show no relationship to mosquito infectivity.

Table 1.

Oocyst counts in *An. dirus* infected by *P. knowlesi* from mixed-stage or crashed cultures

Experiment	*N*	Prevalence	Mean Oocysts	Median Oocysts
Mixed stage cultures				
Expt 1	31	0.07	0.06	0
Expt 2	30	0.10	0.10	0
Expt 3	30	0.10	0.17	0
Crash method				
Expt 1 31h	30	0.07	0.10	0
36h	30	0.17	0.17	0
Expt 2 31h	30	0.13	0.13	0
36h	30	0.10	0.20	0
Expt 3 31h	39	0.10	0.10	0
36h	34	0.09	0.09	0

N=number of mosquitoes dissected; Prevalence=proportion of mosquitoes infected