# Growing Malaria Parasites at a Critical Shaking Speed Mimicking Physiological Flow Reveals New Phenotypes for Invasion Ligands

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**Abstract**

Malaria kills over 600,000 people annually, with all the clinical symptoms being caused by the blood stage of the infection. Malaria parasites invade red blood cells (RBCs), where they grow and multiply until daughter parasites egress to invade new RBCs. This cycle happens primarily in the blood circulation, bone marrow, and spleen, where parasites and RBCs are exposed to flow-generated forces. However, most *in vitro* growth assays are carried out in static conditions, which are a poor mimic for growth in the body. Previously, more dynamic growth conditions were created using orbital shaking platforms, but exploration of the impact of shaking has been limited, and no attempt has been made to understand the forces generated by the resultant fluid motion. For several strains of the deadliest malaria species, *Plasmodium falciparum*, we show that growth under shaking is strongly impacted by shaking speed, vessel geometry and haematocrit. Strikingly, for any given vessel, there is a critical shaking speed at which growth rates are reduced, corresponding to when the RBCs start to aggregate in the centre of the well, at which point the forces that RBCs and parasites are exposed to are comparable to the forces in the microvasculature. By contrast, the growth rate is increased in more turbulent conditions generated by higher shaking speeds. Using a panel of *P. falciparum* lines in which known invasion proteins were disrupted, we show that the critical shaking speed can reveal new growth phenotypes, showing several ligands have greater importance in high wall shear stress conditions.

## 1 Introduction

All clinical symptoms of *Plasmodium falciparum* malaria are caused by the blood stage of the infection, during which the parasites invade and replicate inside red blood cells (RBCs). Blood stage growth requires parasites inside mature infected RBCs, known as schizonts, to egress, releasing the merozoite form of the parasite that goes on to invade uninfected RBCs, a process that typically takes less than a minute [1]. After invasion, the parasite develops into a new schizont containing 26-32 individual merozoites over the next 48 hours [2, 3]. Where parasites develop and invasion occurs in the body is not completely understood; post-mortem analysis of patients with severe malaria has shown that there is a high attachment due to cytoadherence of infected RBCs to capillaries and postcapillary venules in organs [4], and so a key site of invasion is likely in the subsequent vasculature. There is also increasing evidence that a significant proportion of parasite burden may be in the bone marrow [5] and spleen [6, 7]. In these different environments, both parasites and RBCs will be exposed to shear forces resulting from gradients in the velocity, intrinsic to fluid flow in confinement, but *in vitro* studies of parasite growth and invasion have primarily been performed only under static conditions, which is likely to be an imperfect mimic of the *in vivo* environment.

Shaking on an orbital rotation platform is a simple and low-cost method of introducing motion in parasite cultures, and while it does not mimic precisely physiological conditions, it does provide an avenue to explore the impact of fluid-induced forces on parasite growth. Several studies have previously used shaking/ suspension culture for *P. falciparum*, but the results have not always been consistent. Several studies report that shaking produces a higher growth rate [8, 9, 10, 11, 12], although a lower growth rate in shaking has also been shown [13]. Shaking has also been reported to reduce the number of RBCs infected by multiple parasites, suggesting that one impact of the orbital motion is to distribute parasites through the culture [14, 13, 15, 11]. One significant complication is that methodology varies widely between labs and protocols and is not always sufficiently defined in each paper. Vessel shape and size, fluid volume, fluid viscosity, orbital radius, and orbital rotation speed will all impact fluid motion and the resultant mechanical forces generated, and without these being clearly described, it is impossible to draw clear conclusions from the existing literature.

Links between shaking and the physical forces to which parasites and RBCs are exposed have been limited to date. Fluid motion can be characterized by many different metrics, but one critical concept is that flow can be either laminar or turbulent. In laminar flow, fluid moves in smooth, orderly layers with minimal mixing between them. By contrast, turbulent flow is characterized by chaotic, irregular motion, where velocities fluctuate, and significant mixing occurs between fluid layers. For a viscous liquid, both types of flow lead to shear forces, which arise when adjacent layers of fluid move at different speeds. During laminar flow, shear forces primarily arise from the interaction between the fluid and the container walls, where a thin layer of fluid remains stationary relative to the wall. The force exerted on the walls due to fluid flow is called wall shear stress (WSS) [16]. Under these conditions, fluid velocity increases gradually further from the flask wall, creating a smooth velocity gradient. In turbulent flow, however, the velocity profile is much less uniform, leading to more complex shear forces. It is also worth noting that flow conditions at a given speed will vary across a well, due to the non-uniform shear stress field generated by the orbital motion [17], which means complex models are needed even to predict the WSS exerted on the base of the wells [18, 19, 20, 16].

In this study, we systematically explored the effects of shaking on parasite growth in both laminar and turbulent conditions by varying shaking speed, culture volume, flask shape, and haematocrit. This established that shaking can both decrease and increase growth, and identified a critical shaking speed at which the blood first starts to move into suspension, and the calculated WSS acting on the blood is high. By growing a library of genetically modified *P. falciparum* lines at this critical shaking speed, we establish that multiple members of the Erythrocyte Binding Antigen (PfEBA) family and Reticulocyte Binding Protein Homologue (PfRH) family of invasion ligands [21], which are not required for growth under static conditions, are more important when invasion happens in high-shear stress conditions.

# 2 Methods

## 2.1 Plasmodium falciparum culture

*P. falciparum* strains (either the wildtype NF54, 3D7 or Dd2 lines, or genetically modified lines) were cultured in human erythrocytes purchased from NHS Blood and Transplant, Cambridge, UK – ethical approval from NHS Cambridge South Research Ethics Committee, 20/EE/0100, and the University of Cambridge Human Biology Research Ethics Committee, HBREC.2019.40, formal written consent was obtained by the NHS. Unless indicated cultures were kept at a 4% haematocrit (HCT). Cultures were maintained in RPMI 1640 media (Gibco, UK) supplemented with 5 $gl^{-1}$ Albumax II, DEXTROSE ANHYDROUS EP 2 $gl^{-1}$, HEPES 5.96 $gl^{-1}$, sodium bicarbonate EP 0.3 $gl^{-1}$ and 0.05 $gl^{-1}$ hypoxanthine dissolved in 2M NaOH. Culture was performed at 37 $^{o}$C in a gassed incubator or gassed sealed culture container under a low oxygen atmosphere of 1% $O_2$, 3% $CO_2$, and 96% $N_2$ (BOC, Guildford, UK).

## 2.2 Sorbitol synchronisation

Sorbitol synchronisation was performed as described by [22]. Ring stage cultures were pelleted by centrifugation, the supernatant was removed, and the pellet was resuspended at ten times the pelleted volume of 5% D-sorbitol (Sigma-Aldrich), then incubated at 37 °C for 5 mins, during which time later stage parasites (trophozoites and schizonts) rupture. The cells were pelleted by centrifugation and then resuspended in RPMI at twenty times the pelleted volume. The cells were pelleted again and then resuspended in complete media to give 4% HCT.

## 2.3 Preparation of synchronised parasites for growth assay

All lines were cultured in static conditions prior to the assay. For each run of the assay all lines were defrosted at the same time, sorbitol synchronised in the first cycle when the culture was greater than 0.5% rings and then delayed at room temperature to push the egress window to the intended start window. Smears were checked to confirm the culture was predominantly schizonts with few rings. All but 5 ml of the media was removed, and the infected blood was resuspended in the residual liquid, then layered over 5ml of 70% Percoll in a 15 ml tube (Percoll Merck P4937, 10% 10x PBS, 20% RPMI) and centrifuged at 1450 rcf for 11 min with break set to 1 and accelerator to 3. The band of late-stage parasites at the Percoll-media interface was then added to a flask with media and blood at a 4% HCT and incubated in a gassed incubator at 37 °C for 3 h. The Percoll separation was then repeated as above, but this time, the bottom pellet containing ring stage parasites (which had invaded in the previous 3 h) was kept, and then sorbitol synchronised to completely eliminate any late stage parasites, resulting in tightly

synchronised parasites within a 3h development window. The parasitemia was then counted using Giemsa smears and the cultures diluted to a 0.2-0.5% parasitemia at a 4% HCT.

### 2.4 Growth assay

Unless otherwise indicated, three replicates for every condition were grown in parallel to provide technical replicates; the replicates were grown in the same blood. Every two cycles, the samples were split into new blood (at least 50%), ensuring blood from three donors was tested over the course of the experiments, providing further replicates. Every day at ˜3 hours after the initial invasion window the parasitaemia was measured using flow cytometry. 5 $\mu$l of each well was diluted in 45ul of PBS in a 96-well plate. The samples were stained using SYBR Green I (Invitrogen, Paisley, UK) at a 1:5000 dilution for 45 mins at 37 °C. The samples were then run on an Attune NxT acoustic focusing cytometer (Invitrogen) with SYBR green excited with a 488 nm laser (BL1-A) and detected by a 530/30 filter. The parasitemia was measured using the Attune NXT software, for each data set, a plot of SSC-A vs FSCA was used to gate for roughly the size of an RBC, Fig. S3a, and then singlets were gated for in an FSC-H vs FSC-A plot, Fig. S3b. Next, histograms for the BL1-A intensity were used to gate for the SYBR green positive RBCs, the percentage of positive RBCs represents the percentage of infected RBCs, Fig. S3c. Every 48 h, when the culture was ring stage, it was split to give  0.5% parasitemia.

### 2.5 Shaking conditions

Shaking was performed at 45 rpm, 90 rpm or 180 rpm using an orbital shaker (Celltron from Infors HT), which has a 25 mm shaking throw. Comparison of different culture vessels was performed at 4% HCT using a: 50 ml Polystyrene tissue culture treated flask with an area of 25 cm2 (ref 353014 Falcon) with a 5 ml culture volume, a 6-well plate with a 5 ml culture volume, a 24-well plate with a 1ml culture volume or a 96-well plate with a 100 $\mu$l culture volume. Different HCTs were compared in a 6-well plate with a 5 ml culture volume.

### 2.6 Calculating growth

Growth was measured by calculating the Parasite Erythrocyte Multiplication Rate (PEMR). PEMR was defined by [23] as "The ratio between the number of parasites at any point in the Intraerythrocytic development cycle (IDCn) and the number of rings in the IDCn+1.". Custom Python scripts were used for further analysis (available at `10.5281/zenodo.13372478`). As shown previously [13, 24], starting parasitemia can affect growth rate demonstrated by the negative correlation seen when the parasitemia in the initial intra-erythrocytic developmental cycle (IDCn) was plotted against the PEMR of the subsequent cycle (IDCn+1) were plotted, Fig. S4. While the target starting parasitemia for all cycles was  0.5%, splitting inaccuracies meant that there was some variation (64.9% of measurements across all assays had a parasitemia 0.25%-0.75%). The effect is most noticeable when the starting parasitemia is above 1%, therefore, any wells with IDCn¿1% were removed from the data sets (9.7% of measurements had an IDCn¿1% across all assays). Subsequent cycles were unaffected if parasitemia was correctly split to IDNn≺1%. Plots of the PEMR by cycle were assessed by repeat and any data set removed where a very high parasitemia (typically IDCn¿5%) caused subsequent repeats to be very low (2.1% of measurements had an IDCn¿4% across all assays). These wells were manually removed by looking at the PEMR over repeats for every culture tested. The multiple invasion rates were assessed in the ring stage cultures by first using FlowJo (Tree Star, Ashland, Oregon) to plot BL1-A intensity and measured the proportion of reads in peak, Fig. S3.

### 2.7 Data availability

All code, raw data, graphs, and videos supporting the findings of this study are available in the Zenodo repository at `10.5281/zenodo.13372478`.

## 3 Results

### 3.1 Significant agitation is required to prevent RBCs from sedimenting

We began by exploring fluid motion in standard malaria parasite culture conditions - a 6-well plate with each well containing a 5 ml culture volume at 4% haematocrit (HCT) (i.e. 4% w/v RBCs/medium). To understand the conditions that the cells are exposed to in these wells, we filmed how the fluid motion of blood changed as the shaking speed increased, Movie S1 and Fig. S1. At static and low rotational speeds, the RBCs remain sedimented

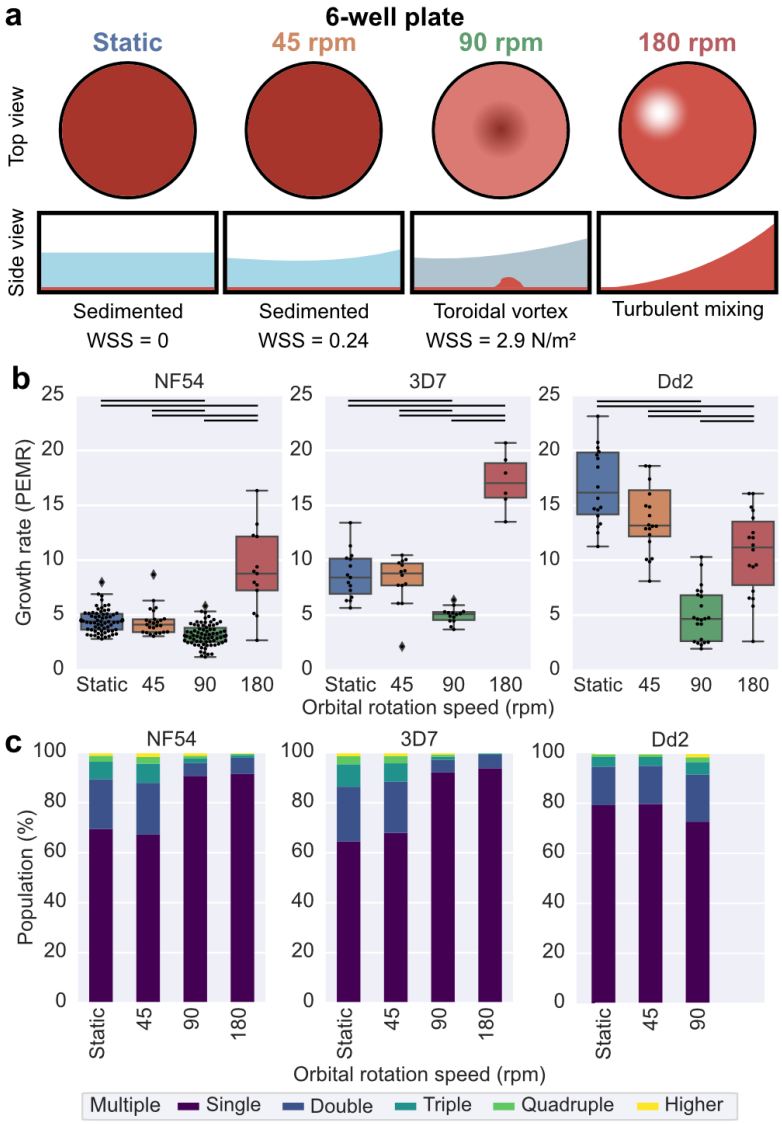


Figure 1: **Shaking speed affects growth rate of wild-type *P. falciparum* lines: NF54, 3D7 and Dd2.** All data was collected for samples cultured in a 6-well plate, 5ml culture volume and a 4% HCT. **(a)** A schematic to represent visually what the motion of the blood looks like at the conditions for which growth was compared. At static, the blood is sedimented at the base of the well; at 45 rpm the fluid just starts to move over the sedimented blood; 90 rpm is just above the critical clustering speed and the RBCs are largely confined to the vortex, and at 180 rpm the conditions are highly turbulent and the RBCs are completely mixed through the medium. The simulated mean WSS are shown for the conditions where the blood is still sedimented. **(b)** The growth rate is shown as the Parasite Erythrocyte Multiplication Rate (PEMR). The data displayed is from all experiments run under the indicated conditions across multiple assays. The lines between conditions indicate the significantly different conditions (t-test) at a greater than 5% significance level. **(c)** Graphs represent the mean frequency of different numbers of parasites in a single RBC for a given speed for each wild-type line. The frequency of single-infected, double-infected, triple-infected, quadruple-infected, and higher-infected rings is shown as a fraction of the total infected ring-stage parasites. The number of rings per erythrocyte was measured using the peak intensity of the ring-stage infected samples and the number of counts in each peak. Data sets without clearly defined peaks were excluded, which is why there is no data for Dd2 at 180 rpm.

at the base of the well. As rotation speed increases the media begins to move above the sedimented RBCs, which results in the cells sedimented at the base of the well being subject to the wall shear stress (WSS). Then, as a critical threshold is surpassed, which we term the *critical clustering transition*, blood begins to cluster in the centre of the well, forming a vortex of RBCs. Further speed increases cause a corresponding increase in the motion of the

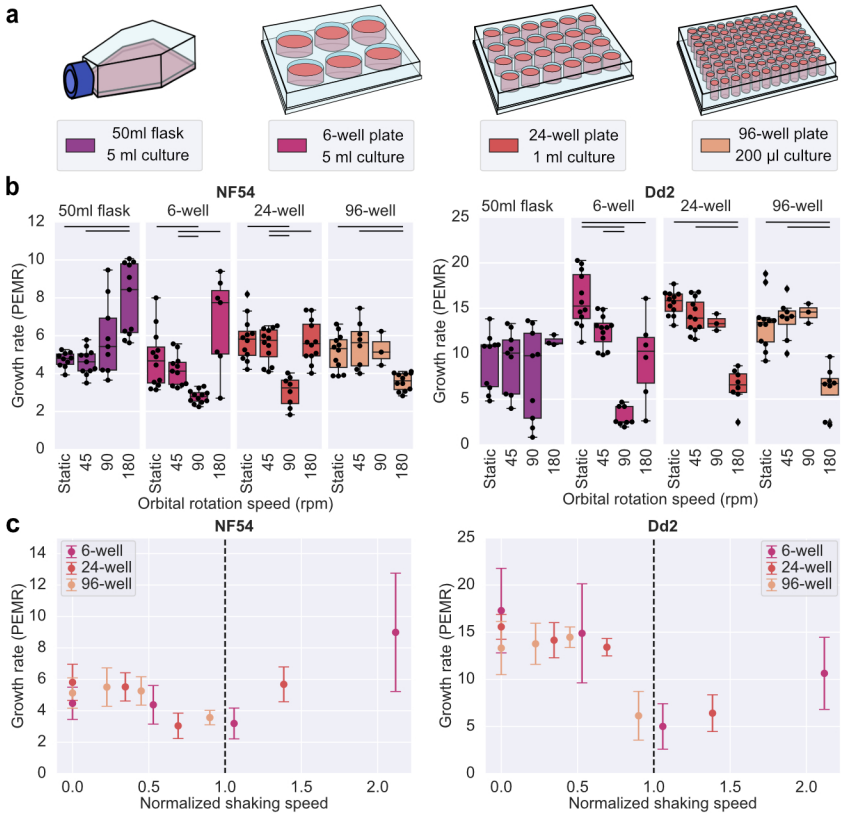


Figure 2: **The type of culture container affects growth rate of wild-type lines of *P. falciparum*. (a)** Schematics illustrate the culture conditions that were compared. **(b)** The wild-type lines NF54 and Dd2 were tested. All cultures were kept at a 4% HCT. The mean growth rate is shown as the parasite erythrocyte multiplication rate (PEMR). The experiment was run for triplicate technical repeats over 6 cycles to give biological replicates; each point shows a PEMR measured for a single invasion cycle for an individual well. The lines between conditions indicate the conditions that were significantly different (t-test or rank sum) at a greater than 5% level of significance. **(c)** Growth rates from (b) for the round wells plotted against the normalised shaking speed. The shaking speed was normalised to the rotation speed at which the blood was first seen to cluster in the centre of the well, Fig. S2a.

vortex structure, and more RBCs become suspended in the laminar flow region surrounding the central vortex. As orbital rotation speed increases further, the RBCs gradually get better mixed into the bulk media, and the flow transitions to become more turbulent; eventually, all the RBCs are in suspension. By testing three shaking speeds, we can capture the different fluid dynamic conditions: 45 rpm, where the fluid is moving over the sedimented blood but the RBCs are not moving, 90 rpm which is just above the critical clustering speed and the RBCs have largely moved into a central vortex, and 180 rpm where the conditions are highly turbulent and the RBCs are completely mixed through the medium, Fig. 1a.

Quantifying the WSS in these round wells under orbital motion is difficult because the fluid motion is non-uniform [17]. To estimate WSS, we used the model presented in [16] and based on [25, 26]. We simulated the mean WSS over time at three points along the bottom of the well (25%, 50%, and 75% between the wall and centre) and reported the mean of these, Fig. 1a and Fig. S2d. WSS increases with orbital rotation speed. For the 6-well plate, the mean WSS at the clustering transition speed (85 rpm) was simulated to be $2.7\,\mathrm{N/m^2}$, which interestingly is comparable to measurements of WSS in human microvessels [27], were late stage parasites sequester [4].

## 3.2 Shaking can both decrease and increase the growth rate of wild-type *P. falciparum* parasites

The growth of three wild-type *P. falciparum* strains (NF54, 3D7 and Dd2) were compared at these three different shaking speeds while keeping all other conditions constant (6-well plate, 5 ml culture volume, 4% HCT). Growth was defined using the Parasite Erythrocyte Multiplication Rate (PEMR), as defined by [23], calculated by dividing parasitemia after invasion (ring stage culture) by parasitemia the day before (trophozoite/early-schizont stage culture). Parasitemia was measured using flow cytometry to calculate the percentage of DNA-positive (ie. infected) RBCs, Fig. S3. The effect of starting parasitemia was carefully controlled for, Fig. S4. The wild-type lines have different PEMRs under static conditions. NF54, which originates from a patient in the Netherlands although genomic analysis suggests it is likely of West African origin [28], had a growth rate PEMR of 4.2 ± 0.2. 3D7, Fig. 1a. 3D7 is a clone of NF54 [28] that has been cultured independently for 30 years and while being genetically very similar [29] has multiple diverged phenotypes [30, 31, 32] including shown here in growth rate with a PEMR of 8.7 ± 0.6, Fig. 1b. Dd2 is a strain of Southeast Asia origin that was chosen for comparison as it has many invasion characteristics distinct from 3D7/NF54 and it had the highest growth rate with a PEMR of 13.7 ± 0.5, Fig. 1b.

These measurements are comparable with previous measurements of growth with qPCR in which 3D7 had a mean growth rate of 8.1 and Dd2 10.5 (assays performed at 3% HCT and 0.02% starting parasitemia) [33]. For all three wild-type lines, there was no significant difference in PEMR between static and 45 rpm, where the RBCs are not in suspension. At 90 rpm, just above the critical clustering speed, the growth rate drops for all lines, while at 180 rpm, the growth rate increases for 3D7 and NF54, but not for Dd2. This shows clearly that shaking does not always produce better growth/invasion rates and that the relationship between growth and shaking is complex.

We quantified the degree to which erythrocytes were infected with multiple parasites based on peaks in the intensity of DNA staining in the ring stage cultures as described previously by [34], Fig. S1d. This was possible as all assays were set up using parasites tightly synchronised to a 3 hour window and measurements were taken at the ring stage, before parasites began DNA replication (see Methods for details). As done previously, we refer to the number of rings measured in each RBC as its multiplicity of infection (MOI), [35]. Previous observations using Giemsa smears have shown up to five rings can be observed in a single RBCs and that different strains have different rates of mean MOI rates [35]. For 3D7 and NF54, there was no difference in the proportion of RBCS with two or more rings between static and 45 rpm, but much lower rates at both 90 rpm and 180 rpm, Fig. 1c, in keeping with previous reports of lower rates of mutiple infected rings when culturing under shaking conditions [14, 15, 10, 11]. This trend was not observed for Dd2, Fig. 1c, but the MOI data for Dd2 is less reliable as its replication cycle is shorter. It is important to note that MOI and growth may be linked, but are not definitively so - despite 90 rpm and 180 rpm having opposing effects on growth for 3D7 and NF54, both speeds reduced mean MOI rates.

### 3.3 The critical clustering speed correlates to a drop in growth rate across different culture conditions.

To further explore the complex relationship between growth rate and shaking conditions, we utilised the fact that changing vessel shape and size changes the resultant fluid flow produced at a given speed. We performed the same growth assay in three additional vessels, Fig. 2a. Filming the changes in the blood motion across shaking speeds for these new conditions showed a consistent fluid flow change as the rotational speed was increased, Movie S2-S4 and Fig. S5. However, the smaller the well, the higher the orbital rotation speed that was required to reach the critical clustering transition. This pattern was observed for all vessels, but the fluid flow was more irregular in the culture flask due to its non-uniform shape, leading to more fluid mixing even at very low shaking speeds. We tested the growth in the different vessels of wild-type lines NF54 and Dd2 at the same range of shaking speeds tested previously, Fig. 2a.

The critical clustering transition speed (when blood began to cluster in the centre of the well, see Fig. S2a) provided a way to normalise the shaking speeds across round well sizes. When the mean growth rates were plotted relative to this normalised orbital rotation speed, there was a clear pattern for both wild-type NF54 and Dd2, with the dip in growth rate occurring close to the clustering transition speed, and growth increasing once normalized rotation increases beyond this transition, Fig. 2c and S2c. Interestingly, simulating WSS for the different well types shows that the shear forces at the critical clustering transition speed are similar in magnitude, Fig. S2d. This is likely because the shear stress is a determining factor in suspending the RBCs. Thus, normalizing to the critical clustering speed will, to some degree, normalize the shear forces experienced by the parasites.

As expected, the culture flask/volume also changed how a given shaking speed affects the multiple invasion rates, Fig. S6a. The switch from high to low MOI rates also corresponds to the clustering transition speed for NF54, Fig. S2b. At this point, the blood starts to move into suspension, reducing the probability that two merozoites that egress from a given schizont will contact and invade the same RBC. Another variable likely affecting how shaking impacts growth is the erythrocyte density in the culture (haematocrit). The growth of 3D7 and NF54 was compared at 2%, 4% and 8% HCT, Fig. S2a. Altering haematocrit changes how a given shaking speed affects growth but not the multiple invasion rate, Fig. S2b. However, in general, a comparison of a single haematocrit as shaking speed increases is consistent with the pattern of decreased growth followed by increased growth. This demonstrates that it is important to keep haematocrit consistent when making comparisons between growth rates under shaking conditions.

### 3.4 Deletion of PfEBA and PfRH invasion ligands changes the impact of shaking on growth

We hypothesised that high WSS leads to lower growth rates because makes it harder for merozoites to attach to RBCs during invasion. Many members of the PfEBA and PfRH family of ligands have linked to the initial steps of invasion [21], and we recently showed several members of these families are key determinants of the strength of attachment between merozoites and RBCs, [36]. We were therefore interested in whether disruption of PfRH and

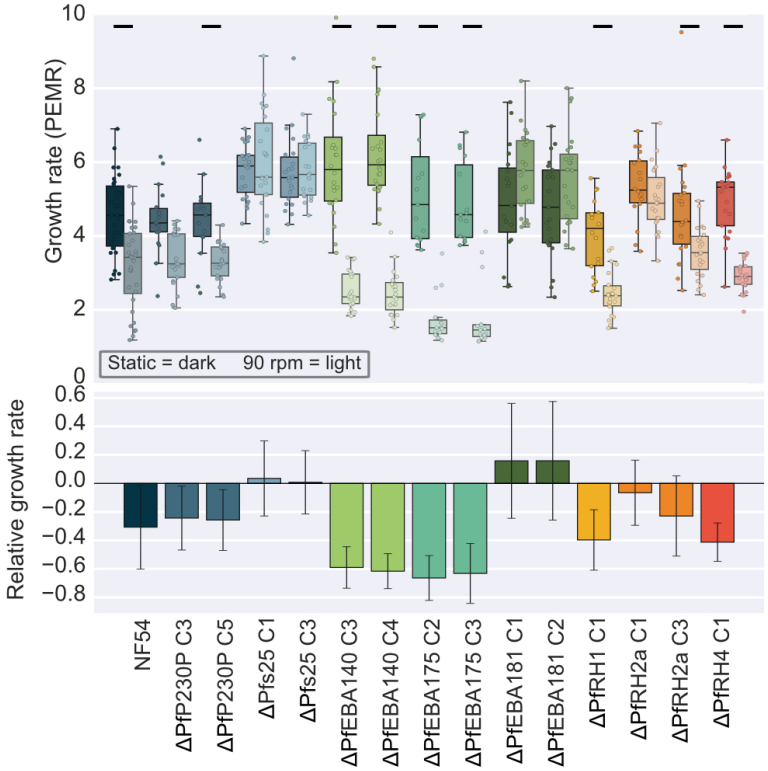


Figure 3: **Knocking out specific invasion ligands shows flow-dependent effects.** Experiments are carried out to compare PfEBA and PfRH knock-out line growth rates under static and detrimental shaking conditions, measuring in static and at 90 rpm; two clones of each line were tested wherever possible. All cultures were grown at 4% HCT in 5 ml in a 6-well plate. The top box plot shows the mean Parasite Erythrocyte multiplication rate (PEMR) of the knock-out lines. The central red line shows the median, with the top and bottom of the box at the 25th and 75th percentiles and the whiskers showing the total range of the data. The bottom bar plot shows the relative difference in growth in shaking compared to static for each line. The error bars show the standard deviation.

PfEBA proteins would impact the change in growth between static and shaking conditions at the critical transition point (6-well plate at 4% HCT and 90 rpm). A panel of PfRH and PfEBA knock-out lines in the NF54 background were tested along with two control knock-out lines constructed in the same manner [36]. The control lines have the gametocyte-specific proteins PfP230P or Pfs25 knocked out, both proteins that have no known role in invasion [37, 38]. Wherever possible, two clones (cX, cY) were tested of a given knock-out to assess the variability between clones. The data for the static growth rates of these lines was previously published [36]. As previously reported by both us and others [39, 40, 41, 42, 43, 44], deletion of these ligands, which are redundant and largely thought to be functionally interchangeable, had no impact on growth under static conditions, although there were some minor differences in growth relative to wildtype NF54 in some knockouts. These minor differences are likely due to epigenetic differences acquired in growth rates or invasion phenotypes of clones during the extended period of culture required to generate transgenic parasites, as has been reported previously [45, 46, 24]. Several of the lines showed higher rates of multiply infected erythrocytes than NF54, including ΔPfs25c1/3, ΔPfEBA181c1/2, ΔPfRH2ac1 (but not c3) and ΔPfRH4c1, Fig. S7.

When PEMR was compared between growth in static conditions and shaking at the critical clustering transition point for the wild-type NF54 line and control lines (ΔPfP230P and ΔPfs25), there was little (less than 30%) or no significant difference, Fig. 3. By contrast, several PfEBA and PfRH knockout lines had a significantly lower PEMR when grown in shaking conditions at the critical clustering transition point compared to static conditions: ΔPfEBA140c3/c4 (c3 2.4-fold lower $p = \prec 0.0000$, c4 2.6-fold lower $p = \prec 0.0000$), ΔPfEBA175c6 (2.5-fold lower $p = \prec 0.0000$), ΔPfRH1c1 (1.7-fold lower $p = 0.0009$) and ΔPfRH4c1 (1.7-fold lower $p = \prec 0.0000$), Fig. 3. It is interesting that for the ΔPfEBA181c1/c2 while there was no significant difference in PEMR between static conditions and 90 rpm; at 90 rpm, the PEMR for ΔPfEBA181c1/c2 was significantly higher than NF54. This suggests that growth under shaking at the critical transition point can reveal growth/invasion phenotypes that are

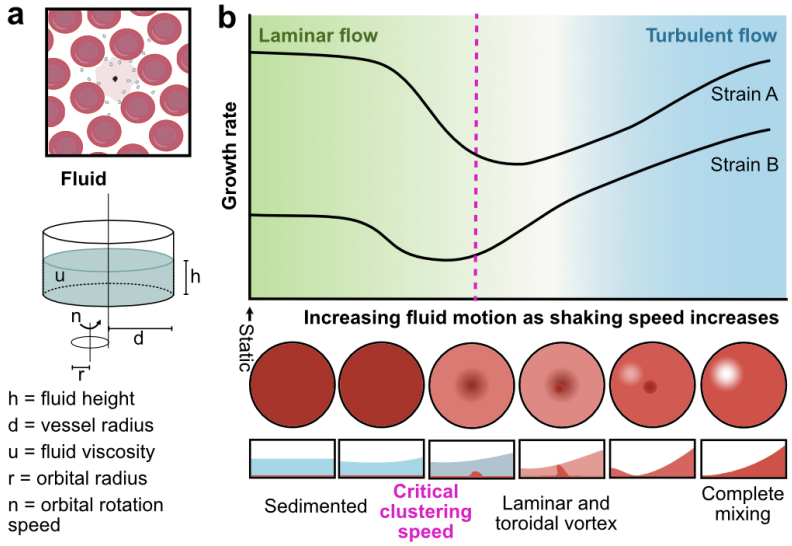


Figure 4: **Summary of effects of shaking on *P. falciparum* growth (a)** Summarize the factors that determine fluid motion when a round well is on an orbital shaker. **(b)** Shows a cartoon of the relation we have shown between increased orbital rotation speed and growth in round wells. Alongside are the observed changes in a fluid motion.

not detectable through static growth assays. In addition, the loss of several of the PfEBA and PfRH proteins seems to be more detrimental to growth under these shaking conditions, meaning these proteins may be more important to binding in conditions of high WSS, which in this assay set up are similar to the conditions that schizonts will experience in the microvasculature.

# 4 Discussion

The data presented here clearly shows that multiple factors must be considered when culturing *P. falciparum* under shaking conditions, particularly the geometry of the culture vessel, Fig. 4a. *P. falciparum* grows very differently while shaking at different orbital speeds, Fig. 1, in different flasks, Fig. 2a, and over different haematocrits, Fig. S8. This is however of more than technical interest, as it relates directly to the conditions that the parasites encounter *in vivo*. We observed that in round wells there is an interesting behaviour of decreased growth at low orbital rotation speeds and then increased growth under higher speeds. While at first glance perhaps unexpected, these changes relate clearly to known elements of fluid mechanics. Fluid motion generated by orbital shaker creates a non-uniform shear stress field even in circular wells [17] and observation of the changes of RBC behaviour in fluid motion showed that different phases can be distinguished as shaking speeds increase, as described above and shown in Fig. S1. The speed where the RBCs transition from sedimented to beginning to cluster in the centre of the well, which differs depending on vessel size/geometry and culture volume, represents a point at which the fluid motion will exert high sheer stress on the sedimented blood. Strikingly, regardless of strain or vessel used, this point also resulted in the lowest growth rates. We propose that this dip in growth occurs because the high WSS at this transition point reduces the rate of successful attachments between merozoites and RBCs during invasion. It has been previously shown that shaking parasites only at the specific time in the life cycle that invasion occurs can directly affect growth [47]. We expand on that finding by showing that disruption of several *P. falciparum* ligands involved in attachment (PfEBAs and PfRHs), further reduce growth rates at this critical transition speed, Fig. 3. At higher shaking speeds, when the blood starts to become suspended and the conditions become more turbulent, growth rates increase and the MOI rate falls, Fig. 2 and S6. It is likely that these turbulent conditions increase the frequency of contact between merozoites and RBCs, which overcomes the detrimental effect of reduced successful attachments due to the increased WSS. We have previously shown that different wild-type lines have different attachment frequencies and detachment forces using an optical tweezer-based assay [36], which would be one reason why different lines are affected to different degrees by a given shaking condition, as shown in Fig. 1 and in existing literature [8, 12, 48]. This relationship is summarised in Fig. 4.

The impact of the geometry of the vessel being used is made clear by the fact that for the specific culture flask we tested, we did not see a drop in growth relative to static conditions, Fig. 2a. This is likely because square flasks produce more mixing than the round wells even at low speeds, Fig. S1, meaning either the lowest speed tested (45

rpm) was too high or the shear forces at the critical clustering speed may be too low to impact the growth rate. This data is in keeping with previous studies where culture conditions are detailed. Where increased growth was reported, flasks were used to grow cultures [15, 10, 11, 12]). Similarly, a previously published example of decreased growth in shaking conditions was performed using a 2 ml culture in a 12-well plate at 125 rpm [13], which based on correlation with our normalized rotational speed data was likely close to the critical clustering point, Fig. 2c and S2a. The data and normalisation presented here provide a framework to design future experiments which can be adapted to the specific set-up in each lab, as it is easy to visually determine the lowest orbital rotation speed at which blood starts to cluster in whatever round, flat-bottom well plate is being used. This provides an easy way of determining conditions that are likely to produce either detrimental (at the critical clustering point) or favourable (above this transition point) growth conditions.

The method presented here also provides a new and simple phenotyping assay to assess factors important to growth and invasion under physiologically relevant shear force conditions. As described above, for a 5 ml culture in 6 well plates or 12.5 mm radius filled with 4% HCT culture and shaken at 90 rpm, the mean shear was $2.5\,\mathrm{N/m^2}$, whilst WSS will vary across the vessels of the circulation system, this is similar to the mean WSS measured in human conjunctival capillaries at $1.5\,\mathrm{N/m^2}$ [27]. As schizonts have been shown to sequester in the microvasculature [4], this means that the range of forces exerted in the wells is in the physiological range, making it particularly interesting that conditions are detrimental to growth. A further adaption of the assay would be to use a doughnut-shaped well, as it has been shown that a doughnut-shaped well produces a much more homogenous pattern of shear stress [17].

Using this new phenotyping assay in combination with PfEBA and PfRH knockout lines, we have established that the invasion ligands PfEBA140, PfEBA175, PfRH1 and PfRH4 are more important for growth under conditions of high shear stress than in static conditions, where none of these lines had growth defects relative to controls [36]. The only one of these ligands previously shown to be more important for growth under shaking culture conditions was PfRH4, as expression of this gene has been shown to be up-regulated in wild-type lines W2mef and Dd2 (but not 3D7) when parasites are grown under constant shaking conditions [12, 48]. When the attachment characteristics of the same panel of PfEBA and PfRH knockout lines were assessed using an optical tweezer-based assay, the only line in which there was a significantly decreased detachment force was ΔPfRH4 [36]. This all points towards the strong attachment of PfRH4 being important to efficient invasion under shaking conditions. It is currently thought that the interactions of PfRHs and PfEBAs during invasion are largely redundant, and this data provides evidence that several proteins may actually be more crucial under high shear forces similar to those experienced *in vivo*. An area for future investigation is whether different ligands could be utilised in different environments in the body, which motivates the further development of a system where invasion can be followed under a more uniform flow that can be more easily quantified and controlled.

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