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Abstract (215 words)

Measurement of parasite proliferation in cultured red blood cells underpins many facets of malaria research, from drug sensitivity assays to assessing the impact of experimentally altered genes on parasite growth, virulence, and fitness. Pioneering efforts to grow *Plasmodium falciparum* in cultured red blood cells revolutionized malaria research and spurred the development of semi-high throughput growth assays using radio-labeled hypoxanthine, an essential nucleic acid precursor, as a reporter of whole-cycle proliferation (Trager and Jensen, 1976; Desjardins et al., 1979). Use of hypoxanthine (Hx) and other surrogate readouts of whole-cycle proliferation remains the dominant choice in malaria research. While amenable to high-throughput inference of bulk proliferation rates, these assays are blind to the underlying developmental and cellular steps of growth in human red blood cells. Modern whole-genome methods promise to reveal much about basic parasite biology, but progress is hindered by limitations of our ability to precisely quantify the specific development and growth events within the erythrocytic cycle. Here we build on standard visual and Hx-incorporation measures of growth by quantifying sub-phenotypes of a rapid proliferator, the multi-drug resistant clone Dd2, from a standard wild type clone, HB3. These data illustrate differences in cycle duration, merozoite production, and invasion rate and efficiency that underpin Dd2's average 2-fold proliferation advantage over HB3 per erythrocytic cycle. The ability to measure refined growth phenotypes can inform the development of high-throughput methods to isolate molecular and developmental determinants of differential parasite growth rates.

Key words: malaria, phenotypes, growth rate, cell cycle, schizogony, invasion, virulence, fitness

1. Introduction

Once the focus of a global eradication campaign, malaria is again entrenched, infecting over 500 million people each year and killing as many as 2 million (Snow et al., 2005). Proliferation of *Plasmodium falciparum* in red blood cells (RBC) is the basis for tertian fever cycles characteristic of this most devastating form of malaria. The classically reported 48h periodicity of fever is due to synchronous parasite development in RBC, corresponding lysis, and massive liberation of newly produced merozoites and toxins. Higher multiplication rates lead to higher parasite biomass which is known to correlate with disease severity in patients from SE Asia (Chotivanich et al., 2000; Timms et al., 2001; Dondorp et al., 2005). In addition to the epidemiological significance of quantifying growth and its relationship to virulence, the role of parasite genes, pathways and regulatory networks in modulating strain-specific growth and proliferation differences remains largely unexplored. Knowledge of these mechanisms will help refine attacks on the parasite through rational drug design, small molecule library screens (Baniecki et al., 2007), and choices of drug combinations. In general, *P. falciparum* parasites are described as progressing from rings (0-24hrs) to trophozoites (24-36) to schizonts (36-48), culminating in the formation of new individual merozoites packaged in mature schizonts that initiate subsequent invasion of uninfected cells. In this asexual cycle a single merozoite leads to the development of 10 or more new merozoites per cell cycle (Roberts and Janovy, 2000). Each stage has been well characterized for gene expression, biochemistry, and morphology (Ben Mamoun et al., 2001), but strikingly little is understood about the natural variation in, and regulation of these processes.

Development of in vitro culturing of *P. falciparum* provided powerful experimental access to this parasite, and was followed closely by development of methods to infer parasite viability and proliferation (Trager and Jensen, 1976; Richards and Maples, 1979; Trager and Jensen, 1997). Visualization by Giemsa-stained blood smears remains the definitive, but cumbersome, method for

discerning parasitemia and viability. Desjardins and co-workers developed a semi high-throughput, quantitative assay using incorporation of an essential radio-labeled nucleic acid precursor, hypoxanthine (Hx) (Desjardins et al., 1979). Subsequent optimizations made this assay the gold standard, effectively mirroring visually determined parasitemias (Chulay et al., 1983; Yayon et al., 1983).

Several other methods have been described for monitoring parasite growth that attempt to mirror the well-established Hx-based assays. While having the advantage of not requiring radioactivity, none of these assays has been adapted to measure the steps of the growth process. Lactate dehydrogenase (pLDH) provides an accurate count of parasites when compared with parasitemia determined by smears (Makler and Hinrichs, 1993). Though useful in ascertaining parasitemias and drug sensitivities (Makler and Hinrichs, 1993; Makler et al., 1993), the reagents are light sensitive, and some precision is lost in determination of dose-concentration effects, especially at low parasitemias. More recently, fluorescent labels such as SYBR Green I, a DNA intercalating dye, have been used to measure parasite growth and show great promise for high-throughput applications (Johnson et al., 2007). The SYBR green method measures parasite density almost instantaneously (Bennett et al., 2004) and gives similar inhibition curves and EC50 values for drug tests when compared to standard radioisotope methods (Smilkstein et al., 2004). As for the standard Hx assays, these methods are currently used to indicate expansion of parasite numbers over complete cycles and consequently serve as ‘black box' readouts that synonomize the events of the growth processes, i.e., the sub-phenotypes that underpin bulk proliferation rates.

Here, we quantitatively dissect the steps of growth in red blood cells by quantifying a series of traits, ranging from classical proliferation using blood smears and Hx incorporation to several nested steps of growth: cell cycle progression and duration, merozoite production, and re-invasion

efficiency. These two parasite clones, Dd2, a fast growing multi-drug resistant (MDR) parasite derived from an Indochina isolate, and HB3, a generally drug sensitive parasite from Honduras. were previously crossed to generate a segregating population of progeny (Wellems et al., 1990),. Traits characterized within this cross provide a baseline for efforts to genetically map determinants of differential growth rates. Genetically defined growth loci will provide an entry point into elucidating genes and mechanisms regulating parasite progression aiding in the development of drugs. Characterization of these traits provides a higher resolution of cellular events contributing to rapid proliferation. Such resolution will be imperative to fully access the power of new whole-genome technologies, chemical genetic screens, and broad-scale gene knockouts and mutagenesis.

2. Materials and Methods

Culture-based assays in *P. falciparum* are significantly influenced by subtle variations in hematocrit, parasite inoculums and life-cycle stage diversity (Chulay et al., 1983; Yayon et al., 1983). Consequently, for these studies culturing protocols, management of parasite stocks, and experimental design were rigorously standardized, including both technical and biological replications. Each experiment was conducted with fresh cultures of HB3 and Dd2 parasites derived from genotyped stock material as a common starting point, eliminating the concern of variation introduced by long-term culturing.

2.1. Parasite culture

Parasites were grown in fresh complete media (CM) [370μM hypoxanthine (Sigma), and 25mM HEPES (Sigma); 0.5% Albumax II (Gibco), 10μg/mL gentamicin (Gibco), and 0.225% sodium bicarbonate (Biosource) in RPMI-1640 medium (Gibco)], O^+ RBC (5.0% hematocrit). Albumax, rather than serum, was used to avoid effect of host variation on parasite growth. All reagents were prepared from the same lots throughout these experiments. Cultures were maintained at constant

pH, 7.2-7.4, and temperature, 37ºC. Culturing involved daily media changes maintaining 1-5% parasitemia to minimize stress and gassing to maintain an atmosphere of 5% CO_2/ 5% O_2/ 90% N_2. This base protocol was slightly modified for specific phenotype assays as described. Temperature effects were not specifically controlled for, however every culture was handled identically throughout the experiments and flasks were at room temperature for only 5 minutes during media changes and gassing.

2.2. Proliferation: Visual growth determination

Five mL cultures containing new rings were synchronized with 5% aqueous D-sorbitol (Fluka BioChemika) in RPMI, and resuspended in 5mL of CM. The next day, parasitemia was reduced by 1/5 with the addition of uninfected RBC and fresh CM. After an additional cycle, parasites were synchronized a second time, and diluted to 1% parasitemia the following day. Assays were initiated on day 5 by distributing 3mL of prepared cultures into each well of 6-well plates (Corning Life Sciences) at 0.1% ring stage parasitemia, and maintained in sealed chambers (Billups-Rothenberg) gassed daily. Smears from each well (technical replicates) were made every 48h (18 per cycle for each parasite clone). Parasitemia was determined and the fold change for each life cycle was calculated. By the 4th cycle, cultures were nearing or reaching their crash level as described by Fairhurst et al. (Fairhurst et al., 2003). Five independent biological replications (n = 5) were generated for each clone. Differences were assessed using an unpaired student *t*-test in all growth studies.

2.3. [^{3}H] Hypoxanthine-incorporation growth assay

Parasites were synchronized twice approximately 44h apart as described above. One cell cycle (approximately 48h) after the second synchronization, 175µL of parasite cultures were distributed into flat-bottom 96-well plates (Corning Life Sciences) at 1.0% parasitemia and 1.0% hematocrit in

low Hx CM (2.94μM). Twelve wells contained RBC controls (no parasites). Dd2 and HB3 were distributed evenly among the remaining wells to eliminate plate-to-plate bias in the comparisons. Duplicate plates were prepared for each experimental series. Plates were incubated in sealed, gassed chambers for 48h. After 48h, 75μL were removed from each well and 100μL of 0.76μCi/mL of [^{3}H] Hx-monohydrochloride (Perkin Elmer) in CM were added. Duplicate plates were removed after 12h or 24h, wrapped in parafilm and frozen at -80ºC to lyse cells. Thawed, lysed cells were transferred onto glass fiber printed filtermats (Wallac) using a cell harvester (Tomtec), mats were dried, and placed in bags (Perkin Elmer) containing 3.5mL of Betaplate Scint fluid (Perkin Elmer), sealed and read on a scintillation counter (1450 Micro beta, Perkin Elmer). Growth values were calculated from counts per minute (CPM) at 12h and 24h. Six to 9 independent biological replications were conducted. The 12h time point captures the parasites in a relatively metabolically inactive early ring stage. The 24h time point captures the late rings transitioning to early trophozoites during which the parasites are highly metabolically active (Yayon et al., 1983).

2.4. Cycle time

Cultures were tightly synchronized by sorbitol on consecutive days and resuspended in 5mL at 1% parasitemia. Experiments were initiated with schizonts stages following the second synchronization. Segmenting schizonts provide a visually definitive, narrow morphological window (approximately 4h) for precise stage identification. Progression of parasite throughout the erythrocytic cycle was monitored using Giemsa-stained smears, and the time between segmenter peaks in sequential cycles. A small volume smear (approximately 50 μl) from each culture was made every 2h, originating from the same culture for each biological replicate, throughout the 12h window spanning the schizont peak for four consecutive life cycles. Cycle time is defined as the duration between the peak schizont parasitemias. This series was conducted independently for three biological replicates with three technical replications of each biological replication (for statistical

analyses, n = biological replicates). Additionally, to ascertain stage divergences throughout the entire cell cycle, we conducted an independent series of smears at 1h intervals spanning 50h for each clone. Time zero was defined as one hour after final synchronization at peak rings. Percent of rings, trophozoites, and schizonts was calculated from each smear. A lowess curve was fitted to the 50 data points using GraphPad Prism software.

2.5. Determining number of merozoites per schizont

Cultures were grown to 20mL at 6-8% parasitemia of predominantly late-stage parasites. Mature schizonts were purified by passage through a VarioMACS separation magnetic column (Miltenyi Biotec) to obtain highly concentrated late-stage parasites following manufacturer's guidelines (approximately 90% parasitemia consisting entirely schizonts) (Balu, 2005). The schizont pellet was resuspended in 200µL of CM and a smear was made and stained with Giemsa. The number of merozoites per schizont was counted using light microscopy (1000X). Only mature schizonts (segmenters) were counted as they have undergone complete schizogony and reflect the final number of new merozoites produced. Schizonts were deemed mature when the parasite vacuolar membrane was indistinguishable from the RBC membrane, contained a single pigmented digestive vacuole, and had clearly separated merozoites with limited overlaps. Fifty to sixty schizonts were counted for each of five independently replicated experiments per clone. The mean, median, mode and range of merozoites per schizont were calculated.

2.6. Merozoite invasion

The schizont pellet from the magnetic synchronization described above was used to initiate invasion assays. A volume of 6.95µL of the schizont pellet was added to 43.05µL of RPMI to yield 1.0% parasitemia. Two-fold dilutions were made from this stock to give starting parasitemias of 0.5%, 0.25%, and 0.125%. Fresh uninfected 5mL RBC cultures in 6-well plates were inoculated with

20μL of volume from each of the dilutions. Cultures were incubated for 12h to allow invasion of RBC. Smears were made and the number of RBC infected with new rings was counted per 2000 RBC. This was repeated for three independent experiments. To account for the effect of varying numbers of merozoites in the schizonts used to initiate the invasion assay, invasion efficiency was calculated using the equation:

$$Efficiency = \frac{\%parasitemia_{new}}{merozoite_{averaqge} * \%parasitemia_{start}} \quad \text{(Equation 1)}$$

3. Results

3.1. Visual counts: Dd2 proliferates faster than HB3 over complete cycles

Giemsa-stained blood smear parasitemias demonstrate the quantitative differences in complete cycle proliferation rates between Dd2 and HB3 (Fig. 1A); Dd2 outgrows HB3 as indicated by fold change. Both parasites exhibit an approximate 6-fold change in the first cycle, initiated at 0.1% rings; however the difference between clones is small. The most pronounced difference is observed in the second cycle during which Dd2 and HB3 expanded by 6.84-fold and 3.03-fold, respectively ($P < 0.01$) (Fig. 1B, left y-axis, Table 1). As parasitemia increased in later stages, there was little difference in per cycle fold change, likely reflecting resource limitations and deteriorating medium quality. Although strain-specific growth at high parasite densities was not the focus of these studies, both cultures exhibited signs of stress at high parasitemia similar to those observed by Fairhurst et al.(Fairhurst et al., 2003) and Dd2 began to crash at day 6 (end of cycle 3). To determine if the starting parasitemia was a factor in parasite fold change, a growth assay was set up with starting parasitemias of 0.125%, 0.25% and 1.0%. As previously observed, no significant differences were observed between Dd2 and HB3 in fold change for the first cycle at any of the three starting parasitemias. However, cycle 2 exhibits significant fold changes differences between parasite lines at starting parasitemias 0.125% and 0.25%, but not 1.0%.

3.2. [^{3}H] Hypoxanthine-incorporation accurately reports difference in proliferation

Hx-incorporation in the first 12h following a full cycle accurately reports growth differences as measured with visual counts (Fig. 1B). Dd2 incorporates significantly more Hx than HB3 at both 12h and 24h ($P < 0.0001$); however, at 24h of Hx incorporation, a much higher growth differential (4.3X) is observed between the two clones. Hx-incorporation reporting of growth is stage dependent as a reflection of nucleic acid production associated with both transcription and DNA replication, a window of approximately 4-8h (Chulay et al., 1983; Yayon et al., 1983). A divergence in the cycle time of the parasite may result in missing the optimal window of incorporation and therefore explains some of the variation in the data at the 24h incorporation time point. This observation suggests that Dd2 has begun DNA replication earlier than HB3 indicating a subtle divergence in cycle time.

3.3. Dd2 erythrocytic cycle is shorter than HB3 due to a shorter ring stage

Highly synchronous parasite cultures were monitored to determine the duration between the peak prevalence of mature schizonts. Both clones diverge from the presumed 48h *P. falciparum* cycle. HB3 parasites transition through the complete cycle at an interval of approximately 50h; Dd2 undergoes a significantly faster RBC cycle (44h, $P = 0.0012$) (Fig. 2A, Table 2). Slight changes in temperature resulting from sample collection may have an effect on progression of the parasite through its erythrocytic cycle. However, these aberrations would affect both parasite lines equally and therefore should not have a bearing on the difference observed between parasites. These differences are robust as the cycle length in each line is stable over several cycles and in biological replicates. In addition, Dd2 does not retain synchrony to the degree of HB3, which attains nearly 90% rings over each of 3 consecutive cycles. To further elaborate the divergence in the cell cycle between these clones, an additional experiment was conducted at 1h resolution over the complete

cycle to highlight the successive increases and decreases of each stage and to identify the point of divergence (Fig. 2B). The shorted cycle of Dd2 is due to a more rapid transition from rings to trophozoites.

3.4. Dd2 generates more merozoites per schizont than HB3

Mature schizonts of Dd2 and HB3 contain, on average, 18 and 16 merozoites, respectively (Fig. 3). Only mature segmenting schizonts were counted to ensure that schizogony was completed. These subtle differences were stable over 8-10 biological replicates. For each replicate Dd2 generated schizonts containing between 8-24 merozoites, while HB3 produced 8-20 (Table 3); Dd2 always produced a higher maximum than HB3. Comparisons of synchronized and unsynchronized cultures generated the same distributions (data not shown) demonstrating that if only segmenters are counted, merozoite number is stable and not a reflection of schizont maturity or culture conditions. This confirms segmenters have completed schizogony. A large majority (85-90%) of merozoites counted for both clones were even numbers. To confirm these observations, slides were blinded and recounted by 3 different individuals. Of 140 schizonts meeting the criteria for counting, only 9 (6.4%) contained odd numbers of merozoites. These results suggest that the rare odd-numbered counts may reflect counting errors due to overlapping nuclei not discernable by light microscopy. Likewise, the confirmed preponderance of even numbers indicates that counts were accurate.

3.5. Dd2 merozoites invade new RBC at a higher rate than HB3

Dd2 consistently reinvades at a rate nearly 3X greater than HB3 (Fig. 4A, B). This difference does not change if multiple parasites in a single RBC are counted as a single invasion event or if each new ring is counted separately, indicating that that rate of multiple invasions of a single RBC does not vary between these clones. We observe a decrease in the number of multiply invaded RBCs when cultures are shaken; however, the difference in invasion remains consistent between parasite

lines (data not shown). Because Dd2 produces more merozoites per schizont than HB3, an invasion efficiency rate was calculated using Equation 1. The maximum invasion efficiency of Dd2 per single schizont is about 35% compared to 25% for HB3 (Fig. 4B). Invasion is impacted by parasite density (starting parasitemia), but no difference is observed between parasites lines.

4. Discussion

Strain-specific proliferation rates can highlight components of differential virulence and fitness of malaria parasites. Dd2 and HB3 originate from isolates from Indochina and Honduras, respectively. More importantly, these clones represent vastly different drug selection histories: Dd2 is a fast-growing, MDR parasite from a region where MDR is widespread, while HB3 is a generally drug sensitive, wild type line. Important questions about the maintenance and spread of drug resistant malaria include possible relationships between drug selection and growth rates. One tool to address such questions is quantitative trait loci (QTL) mapping using the segregating progeny population of a cross between HB3 and Dd2 to generate a list of candidate genes that could contribute to parasite growth. However, to maximize the power of QTL mapping and other genomic approaches, it is first essential to understand the fine detail of their phenotypic differences.

Methods of measuring parasite growth generally rely on a surrogate indicator, typically Hx, calibrated to parasitemia as determined by blood smears. Despite attempts to clarify and control extraneous factors in Hx-incorporation, subtle variations in culture parameters lead to extensive biological noise. Consequently, growth assays have evolved to blur this variation by using asynchronous cultures and extending assays over complete cycles to minimize the impact of stage-specificity and within-cycle variation. These sources of variation are biologically relevant. For example, standard Hx-based assays provide robust inference of proliferation and viability and are easily adapted to semi-high-throughput applications, e.g. for drug concentration-effect curves.

However, this approach ignores the fine-tuned underlying cell biological and developmental steps that influence parasite proliferation. Stage-specific events, most notably invasion, are especially susceptible to misinterpretation using standard bulk Hx assays. Early efforts systematically examined the parameters influencing Hx reporting of parasite proliferation (Chulay et al., 1983; Yayon et al., 1983); we build on this approach to score nested traits that contribute to clone-specific growth rates: cell cycle duration, merozoite production, and merozoite re-invasion. The difference in proliferation rates between Dd2 and HB3 over complete cycles results from Dd2's shorter cycle time, production of more merozoites per schizogony, and higher invasion rates.

Visual counts and Hx-incorporation confirm our anecdotal observations that Dd2 is a faster-proliferating parasite (Fig. 1). Hx-incorporation in the 12h following a complete cycle of growth effectively recapitulates the smear-determined growth differences; however, the 24h incorporation window reported a much larger growth advantage for Dd2. Though both methods identify differences between Dd2 and HB3, it is not surprising that the Hx-incorporation counts at 24h do not match fold change measured with smears because starting parasitemias were different. This is consistent with observations that Hx-incorporation accurately reflects visual counts only in a narrow range of parameters (0.4% or less parasitemia after 48h of growth and 18h of incorporation) (Chulay et al., 1983). The majority of Hx-incorporation occurs in the trophozoite stage and reflects the rapid increase in DNA replication and transcription (Desjardins et al., 1979; Chulay et al., 1983; Yayon et al., 1983; Llinas et al., 2006). Llinas et al. demonstrate remarkably well conserved strain-specific *P. falciparum* transcription profiles only after the data are normalized to account for cycle time divergence; specific cycle durations are not reported (Llinas et al., 2006).

P. falciparum is famous for its 48h erythrocytic cycle and associated cyclical fevers (Roberts and Janovy, 2000). Variations in the length of the cell cycle could contribute to biomass accumulation

over many asexual cycles. We find a significant difference in the duration of the asexual cycle of HB3 and Dd2 in RBC as measured from peak schizont prevalence (Fig. 2). Closer examination at 1h resolution attributes this difference to a decreased ring to trophozoite transition time in Dd2 (Fig. 2B). Janse et al. recently knocked out one of two eukaryotic elongation factors (eef1α) in *P. berghei* resulting in a 2h to 4h increase of the overall cycle time (a 10-20% increase) associated with the G1 phase. The S/M phase does not show any additional change in overall cycle time indicating the difference in cycle time is occurring in the early part of the cycle (Janse et al., 2003). Transitions between stages are finely highly regulated, although mechanisms are not well characterized in malaria parasites. Progression through the yeast cell cycle is controlled by cyclins and cyclin dependent kinases (cdks), for which several orthologs have been identified in *Plasmodium* (Doerig et al., 2002); furthermore, microarray analysis pointed to a number of genes that may be involved in regulating the *P. falciparum* cell cycle, including cyclins and kinases (Doerig et al., 2002; Le Roch et al., 2003). The phenotypes described here do not reveal mechanisms, but highlight candidate biological process in which regulatory variation can be tested using genetic mapping or gene manipulation approaches.

It is generally stated that *P. falciparum* schizonts produce 8 – 24 merozoites, but few reports have determined this number. In a recent study, Hayward et al. proposed that growth rate differences in genetically modified parasites result from a fitness impact of mutations introduced in *pfmdr1,* however they ruled out differential merozoite production as contributing to growth differences in these lines (Hayward et al., 2005). We observe a significant difference in merozoite production between HB3 and Dd2 which produce approximately 16 and 18 merozoites per schizont, respectively (Fig. 3). For each independent experiment, the Dd2 maximum always exceeds HB3 (24 vs 20 merozoites). The number of merozoites produced is consistent with asynchronous DNA replication such that each nuclear division is independent of others, i.e. synchronous replication

would yield 2, 4, 8, 16, or 32 merozoites. Furthermore, predominantly even numbers of merozoites are generated by both clones; 85 - 90% of schizonts contain an even number of merozoites, as confirmed by blinded replicate counts. We suggest that the occasional odd-number count is likely counting error. Read et al. reported odd and even nuclear bodies, however their approach relied on counting all schizonts with more than two nuclear bodies from slides of unsynchronized cultures. They did not impose our strict criterion of counting only fully segmented schizonts indicating schizogony had been completed (Read et al., 1993).

Replication of DNA begins in *P. falciparum* as early as 26h and ends around 37h post-invasion (Chulay et al., 1983; Yayon et al., 1983; Arnot and Gull, 1998). Nuclear divisions occur in a single cell (syncytium) and are distinct from partitioning of components of the new individual cells; consequently, the final number of merozoites is not necessarily a direct reflection of DNA replication, but also includes additional cellular events of merogony. Merogony, while not well-studied in *P. falciparum*, has been carefully examined the apicomplexan parasite, *Theileria parva* (Shaw and Tilney, 1992). This process begins with the interconnecting of rhoptries, mitochondria, nucleus, and outer plasma membrane in an ordered and manner. Individual merozoites bud from the nuclear mass as membranes form around the interconnected organelles, generating a host cell with large numbers of free mature merozoites capable of invading RBC.

Dd2 produces more newly infected RBC than HB3 (Fig. 4A) as determined by counting new rings. While it is not feasible to conduct high-throughput studies by direct visualization as done here, similar parameters could be adapted to a flow cytometric analysis to detect newly invaded cells (ring stages) from a starting population of concentrated, late-stage schizonts. When the differences in average starting merozoite numbers are accounted for, the Dd2 growth advantage is maintained, but at a lower level (Fig. 4B). Invasion rates are equally sensitive to starting parasitemia in both

clones, an observation that is consistent with reports for *P. chabaudi chabaudi* (Chimanuka et al., 1997) in which lower starting parasitemia results in greater invasion efficiency.

Indirect assessments of growth using various reporter systems suffer from limitations in their ability to decipher sub-phenotypes that drive parasite proliferation. Hx-incorporation has been the workhorse of dose response and invasion assays to date, however this approach has not been effectively adapted to infer sub-cycle traits. The data presented here suggest that quantitative dissection of the growth process is necessary to fully understand the effects of drugs or other manipulations on parasite lines. Refinement of these methods for high-throughput applications are needed in parallel with the emergence of powerful genomics tools for assessing these mechanistic steps of growth. For example, in screening compound libraries for antimalarial activity, it will be useful pinpoint the specific events of the erythrocytic cycle that are inhibited. In this study, we outline subtle but quantifiable strain-specific differences in traits underlying proliferation rate of *P. falciparum* in RBC. Dd2's faster growth can be attributed to a decreased cycle time and increased merozoite production and invasion rates. These differences in growth between Dd2 and HB3, parents of a genetic cross, suggest that progeny from this cross will differentially inherit growth traits, facilitating QTL analysis to find candidate growth genes. Our observation that laboratory cultured MDR parasites derived from S.E. Asia, including Dd2, tend to incorporate Hx at high rates could suggest a link between parasite growth and fitness and virulence. By further understanding the genes and mechanisms controlling growth and the relationship they have with drug response, we will be able to further characterize the mechanisms of drug resistance.

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Legends to Figures

Fig. 1. Dd2 and HB3 proliferation rates by visual smears and [^{3}H]-hypoxanthine. (A) Determination of parasitemia via Giemsa-stained smears was used to monitor parasite proliferation (bulk growth) per complete cycle (approx. every 2 days). Parasitemia is determined from 1000 RBC. (B) The left y-axis denotes fold-change in parasitemia between days 2 and 4 (cycle 2) for Dd2 (hashed bars) and HB3 (white bars) derived from the same data presented in figure 1A. The right y-axis shows counts per minute (CPM) of Hx-incorporation in Dd2 and HB3 at 12h and 24h post-labeling (labeling occurs after one complete 48h cycle). The starting parasitemia for fold-change is similar to that used for Hx-incorporation. At 12h the Hx-incorporation method best approximates the parasitemia determined by fold change. All data is based on 5-6 independent replicates. Error bars represent SEM. Unpaired *t*-tests indicate significance between parasite lines: **** $P<0.0001$, *** $P<0.001$, ** $P<0.01$, * $P<0.05$.

Fig. 2. Cycle time and stage duration comparisons of Dd2 and HB3. (A) Three biological replicates consisting of Giemsa-stained smears counted 1000 RBC every 2h over a 12h peak period for three consecutive cycles each. The average cycle time for Dd2 and HB3 is 44.1h and 49.7h respectively. Bars represent three biological replicates. Error bars are SEM. An unpaired *t*-test gives a $P<0.005$. (B) Hourly smears taken throughout the erythrocytic cycle (50h) show a decreased time in the ring and trophozoite stages for Dd2 compared to HB3.

Fig. 3. Merozoites generated in Dd2 and HB3 schizonts. (A) Magnetically purified schizonts are viewed by light microscopy to determine the number of merozoites produced per schizont. Five replicates consisting of a minimum of 50 schizonts are counted. Error bars represent the SEM. Unpaired *t*-tests comparing merozoite number between Dd2 and HB3 give a $P<0.05$ for 10, 20, and 22. (B) Representative images of Dd2 and HB3 countable mature schizonts are shown.

Fig. 4. Invasion in Dd2 and HB3. (A) The number of new rings per 2000 RBC is determined by light microscopy. Fold change differences between Dd2 and HB3 in invasion are determined for all replicates at each starting parasitemia giving an average overall fold change of 2.99X. (B) The invasion efficiency of merozoites is calculated using Equation 1. Data points represent a minimum of 3 biological replicates. Error bars are SEM. Unpaired *t*-test gives $P<0.05$ where indicated by *.