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Quantitative dissection of clone-specific growth rates in cultured malaria parasites

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Abstract

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 (Hx), an essential nucleic acid precursor, as a reporter of whole-cycle proliferation (Trager and Jensen, 1976; Desjardins et al., 1979). The isotopic Hx assay remains the standard quantitative growth assay with which new non-radioactive procedures based on fluorescent DNA dyes or ELISA are compared. All of these readouts are surrogate reporters of changes in bulk parasitemias, reflecting proliferation over entire asexual reproductive cycles. While quantitatively robust and amenable to semi-high-throughput applications, these methods are blind to the underlying developmental and cellular events of growth in human red blood cells. Modern whole-genome tools including gene knockouts, mutagenesis and small molecule screens promise to reveal much about basic parasite biology; however methods to precisely quantify the within-cycle growth process are needed. Here we elaborate on the classical growth index, i.e. changes in parasitemia, by quantifying sub-phenotypes of a rapid proliferator, the multi-drug resistant clone Dd2, and 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 two-fold proliferation advantage over HB3 per erythrocytic cycle. The ability to refine growth phenotypes will inform the search for molecular determinants of differential parasite growth rates and broaden our understanding of killing mechanisms and cellular targets of antimalarial drugs.

Keywords

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 (RBCs) is the basis for tertian fever cycles

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characteristic of this most devastating form of malaria. The classically reported 48 h periodicity of fever is due to synchronous parasite development in RBCs, corresponding lysis and large scale liberation of newly produced merozoites and toxins. Increasing multiplication rates generate higher parasite biomass, which is known to correlate with disease severity in patients from South East Asia (Chotivanich et al., 2000; Timms et al., 2001; Dondorp et al., 2005). In addition to illuminating mechanisms of growth rates and their contribution to virulence, much remains to be explored concerning the role of parasite genes, pathways and regulatory networks in modulating strain-specific growth and proliferation. Knowledge of high-resolution growth phenotypes will contribute to fresh attacks on the parasite through targeted drug design and choice of drug combinations, and will be especially powerful when combined with genome-scale gene knockout studies and high-throughput screens (HTS) to assess the cellular targets of structurally diverse molecules (Baniecki et al., 2007).

In general, *P. falciparum* parasites are described as progressing from rings (0–24 h) to trophozoites (24–36 h) to schizonts (36–48 h), 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 methods provided powerful experimental access to *P. falciparum* and was closely followed 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 parasite 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, making it the workhorse of drug sensitivity assays for more than 25 years (Chulay et al., 1983; Yaron et al., 1983).

New non-radioactive methods show great promise in approximating the quantitative precision of the well-established Hx-based assays while being comparatively simpler and less expensive. For example, fluorescent DNA stains such as SYBR Green I and 4',6-diamidino-2-phenylindole (DAPI) are amenable to high-throughput inference of parasite growth inhibition in the presence of antimalarial drugs and diverse small molecules (Baniecki et al., 2007; Johnson et al., 2007). The SYBR green method measures parasite density almost instantaneously (Bennett et al., 2004) and gives similar inhibition curves and IC₅₀ values for drug tests compared with standard radioisotope methods (Smilkstein et al., 2004). Notably, assays relying on each of these surrogate growth measures, including Hx incorporation, have been developed as reporters of inhibition of parasite numbers over entire cell cycles. Consequently they serve as 'black box' readouts that necessarily synonomize the steps of growth, i.e., the sub-phenotypes that underpin bulk proliferation rates. Much can be gained from modifying these promising assays to develop a more detailed picture of parasite viability and the cellular events that comprise overall growth rates. For example: What steps of the erythrocytic growth cycle are being targeted by drugs or small molecules? What is the dynamic of parasite cell death underlying a dose-response curve? What is the cellular basis of differential growth rates?

Here, we dissect the steps of growth in RBCs by quantifying a series of traits in 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. In addition to the classical growth measures of proliferation using blood smears and Hx incorporation, we examine several

nested steps of growth including cell cycle progression and duration, merozoite production and re-invasion efficiency. These two parasite lines were previously crossed to generate a segregating population of progeny (Wellems et al., 1990). These refined growth traits can be characterized within this cross to genetically map loci carrying determinants of differential growth rates. Furthermore, characterization of these traits provides a higher resolution of cellular events contributing to rapid proliferation and to cell inhibition upon exposure to drugs. 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) [370 μ M Hx (Sigma) and 25 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Sigma); 0.5% Albumax II (Gibco), 10 μ g/mL gentamicin (Gibco) and 0.225% sodium bicarbonate (Biosource) in RPMI-1640 medium (Gibco)], O⁺ RBCs (5.0% hematocrit). Albumax, rather than serum, was used to avoid the effect of host variation on parasite growth. All reagents were prepared from the same lots throughout these experiments. Cultures were maintained at a constant pH (7.2–7.4) and temperature (37°C). Culturing involved daily media changes maintaining 1–5% parasitemia to minimize stress and gassing to maintain an atmosphere of 5% CO₂/5% O₂/90% N₂. 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 min during media changes and gassing to minimize cooling.

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 RBCs 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 3 mL 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) which were gassed daily. Smears from each well (technical replicates) were made every 48 h (18 per cycle for each parasite clone). Parasitemia was determined and the fold change for each lifecycle was calculated. By the fourth cycle, cultures were nearing or reaching their crisis level as described by Fairhurst et al. (2003). Five independent biological replications ($n = 5$) were generated for each clone.

2.3. [³H] Hypoxanthine-incorporation growth assay

Parasites were synchronized twice approximately 44 h apart as described above. One cell cycle (approximately 48 h) after the second synchronization, 175 μ L 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 (2.94 μ M). 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 48 h. After 48 h, 75 μ L were removed from each well and 100 μ L of 0.76 μ Ci/mL of [3 H] Hx-monohydrochloride (Perkin Elmer) in CM were added. Duplicate plates were removed after 12 h or 24 h, wrapped in parafilm and frozen at -80°C 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 3.5 mL 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 12 h and 24 h. Six to nine independent biological replications were conducted. The 12 h time point captures the parasites in a relatively metabolically inactive early ring stage. The 24 h 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 5 mL of CM at 1% parasitemia. Experiments were initiated with schizont stages following the second synchronization. Segmenting schizonts provide a visually definitive, narrow morphological window (approximately 4 h) for precise stage identification. Progression of parasites 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 μ l) from each culture was made every 2 h, originating from the same culture for each biological replicate, throughout the 12 h 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 1 h intervals spanning 50 h for each clone. Time zero was defined as 1 h 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 version 4.0.

2.5. Determining number of merozoites per schizont

Cultures were grown to 20 mL 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 of schizonts) (Balu, 2005). The schizont pellet was resuspended in 200 μ L of CM and a smear was made and stained with Giemsa. The number of merozoites per schizont was counted using light microscopy (1,000 X magnification). 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 60 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 6.95 μ L of the schizont pellet was added to 43.05 μ L of RPMI to 9 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 5 mL RBC cultures in 6-well plates

were inoculated with 20 μ L vol. from each of the dilutions. Cultures were incubated for 12 h to allow invasion of RBCs. Smears were made and the number of RBCs infected with new rings was counted per 2,000 RBCs. 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:

$$\text{Efficiency} = \frac{\% \text{ parasitemia}_{\text{new}}}{\text{merozoite}_{\text{average}} * \% \text{ parasitemia}_{\text{start}}} \quad (\text{Equation 1})$$

2.7. Statistics

Significant differences were determined using an unpaired Student's *t*-test in all growth studies and defined as having a $P < 0.05$.

3. Results

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

The quantitative differences in complete cycle proliferation rates between Dd2 and HB3 are demonstrated by Giemsa-stained blood smears (Fig. 1A); Dd2 outgrows HB3 as indicated by fold change. Both parasites exhibit an approximate six-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 the 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. (2003) and Dd2 began to reach crisis stage 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 exhibited significant fold change differences between parasite lines at starting parasitemias 0.125% and 0.25%, but not 1.0%.

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

Hx-incorporation in the first 12 h following a full cycle accurately reports growth differences as measured with visual counts (Fig. 1B). Dd2 incorporates significantly more Hx than HB3 at both 12 h and 24 h of incorporation ($P < 0.0001$); however, at 24 h of Hx incorporation, a much higher growth differential (4.3-fold) is observed between the two clones. Most Hx-incorporation occurs in a window of approximately 4–8 h reflecting stage dependent nucleic acid production associated with both transcription and DNA replication (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 explain the wider variation at the 24 h incorporation time point. This observation suggests that Dd2 begins 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 duration of the erythrocytic cycle. Both clones diverge from the presumed 48 h *P. falciparum* cycle. HB3 parasites transition through the complete cycle at an interval of approximately 50 h; Dd2 undergoes a significantly faster RBC cycle (44 h, $P = 0.0012$) (Fig. 2A, Table 2). Slight changes in temperature resulting from handling parasite cultures likely has a small effect on progression of the parasite through its erythrocytic cycle; however, these aberrations would affect both

parasite lines equally and should have no bearing on the difference observed between parasite clones. Clonal differences in cycle length are consistent and stable over several cycles as observed in biological replicates. Moreover, Dd2 does not retain synchrony to the degree of HB3, which attains nearly 90% rings over each of three consecutive cycles. To further elaborate the divergence in the cell cycle between these clones, an additional experiment was conducted at 1 h resolution over the complete cycle. This experiment highlights the successive increases and decreases of each stage and identifies the point of divergence; the shorter cycle of Dd2 is due to a more rapid transition from rings to trophozoites (Fig. 2B).

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 eight to 10 biological replicates. For each replicate Dd2 generated schizonts containing between eight to 24 merozoites, while HB3 produced eight to 20 (Table 3); Dd2 always produced a higher maximum than HB3. Comparisons of synchronized and unsynchronized cultures generated the same distributions (data not shown), and demonstrate that the merozoite number is stable and not a reflection of schizont maturity or culture conditions, and that segmenters have completed schizogony. In both clones, a large majority (85–90%) of schizonts generate even numbers of merozoites. To confirm these observations, slides were blinded and recounted by three different individuals. Of 140 schizonts meeting the criteria for counting, only nine (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 RBCs at a higher rate than HB3

Dd2 consistently reinvades at a rate nearly three times 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. When cultures are shaken, we observe a decrease in the number of multiply invaded RBC; 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 with 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 Indochina and Honduras isolates, 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 the parasites' 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. drug concentration-effect curves. However, this approach ignores the fine-tuned underlying cell biology 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 schizont 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 12 h following a complete cycle of growth effectively recapitulates the smear-determined growth differences; however, the 24 h 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 24 h do not match fold changes 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 48 h of growth and 18 h 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. (2006) 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.

Plasmodium falciparum is famous for its 48 h erythrocytic cycle and associated cyclical fevers (Roberts and Janovy, 2000). Variations in the length of the cell cycle could contribute to a divergence of biomass accumulation over many asexual cycles. We find a significant difference in the duration of the asexual cycle of HB3 and Dd2 in RBCs (Fig. 2). Closer examination at 1 h resolution attributes this difference to a decreased ring to trophozoite transition time in Dd2 (Fig. 2B). Janse et al. (2003) recently knocked out one of two eukaryotic elongation factors (eef1 α) in *Plasmodium berghei* resulting in a 2–4 h increase of the overall cycle time (a 10–20% increase) associated with the G1 phase. The DNA synthesis/mitosis phase of the cell cycle 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. Transitions between stages are finely regulated, although these 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 a 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 eight to 24 merozoites, but few reports have determined this number. In a recent study, Hayward et al. (2005) proposed that

growth rate differences in genetically modified parasites result from a fitness impact of mutations introduced in *pfmdr1*. Using merozoite number as a growth criteria they ruled out differential merozoite production as contributing to growth differences in these lines. 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 versus 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 two, four, eight, 16 or 32 merozoites. Furthermore, predominantly even numbers of merozoites are generated by both clones as confirmed by blinded replicate counts. We suggest that the occasional odd number of schizonts is likely to be a counting error. Read et al. (1993) reported odd and even counts of 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.

Replication of DNA begins in *P. falciparum* as early as 26 h and ends around 37 h 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 may also be impacted by the cellular events of merogony. Merogony, while not well-studied in *P. falciparum*, has been carefully examined in the apicomplexan parasite, *Theileria parva* (Shaw and Tilney, 1992). Individual merozoites bud from the nuclear mass and interconnect with rhoptries, mitochondria and the outer plasma membrane in an ordered manner. Membranes form around the interconnected organelles, generating a host cell with large numbers of free mature merozoites capable of invading RBCs.

Dd2 produces more newly infected RBCs 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 *Plasmodium 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 to pinpoint the specific events of the erythrocytic cycle that are inhibited (Baniecki et al., 2007).

In this study, we outline subtle but quantifiable strain-specific differences in traits underlying proliferation rates of *P. falciparum* in RBCs. 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 South East Asia, including Dd2, tend to incorporate Hx at high rates could suggest a link between parasite growth, fitness and virulence. A mechanistic understanding of the genes controlling growth will further define mechanisms of drug resistance and the broader impact of past drug selection on parasite biology.

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Fig. 1. Dd2 and HB3 proliferation rates by visual smears and [³H]-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 1,000 RBCs. (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 Fig. 1A. The right y-axis shows counts per minute (CPM) of Hx-incorporation in Dd2 and HB3 at 12 h and 24 h post-labeling (labeling occurs after one complete 48 h cycle). The starting parasitemia for fold-change is similar to that used for Hx-incorporation. At 12 h the Hx-incorporation method best approximates the parasitemia determined by fold change. All data is based on five to six independent replicates. Error bars represent the S.E.M. 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 1,000 RBCs every 2 h over a 12 h peak period for three consecutive cycles each. The average cycle time for Dd2 and HB3 is 44.1 h and 49.7 h, respectively. Bars represent three biological replicates. Error bars are the S.E.M. An unpaired *t*-test gives a $P < 0.005$. (B) Hourly smears taken throughout the erythrocytic cycle (50 h) show a decreased time in the ring and trophozoite stages for Dd2 compared with 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 S.E.M. Unpaired *t*-tests comparing merozoite numbers between Dd2 and HB3 give a $P < 0.05$ for 10, 20, and 22 merozoites per schizont. (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 2,000 RBCs 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 change of 2.99-fold. (B) The invasion efficiency of merozoites is calculated using Equation 1. Data points represent a minimum of three biological replicates. Error bars are the S.E.M. Unpaired *t*-test gives a *P* < 0.05 where indicated by *.

Table 1
Fold change per erythrocytic cycle calculated from blood smear parasitemias.

Cycle ^a	Dd2		HB3		P
	Mean	S.E.M.	Mean	S.E.M.	
1	6.481	0.289	8.733	1.358	0.1367 ^{ns}
2	6.838	0.713	3025	0.324	0.0006 ^{***}
3	3.043	0.304	3.084	0.412	0.8465 ^{ns}
4	20.81	2.75	9.329	1.402	0.0032 ^{**}

^a Cycle: consecutive 48 h periods over 8 days.

^{ns} not significant

*** $P < 0.001$

** $P < 0.01$

Table 2

Summary of strain-specific differences in erythrocytic cycle duration.

	Dd2	HB3
mean	44.1 h	49.7 h
S.E.M.	1.06	1.20
median	43 h	51 h
range	39–48 h	42–54 h
amplitude ^a	80.8%	88.8%

^aThe amplitude indicates the average percent of schizonts per total number of infected red blood cells at the peak of the cycle as a measure of synchronicity ($n = 3$).

Table 3Distribution of merozoites^a in late-stage schizonts.

	Dd2	HB3
mean	17.52	14.83
S.D.	± 1.08	± 0.95
median	17.76	15.08
mode	18	16
range	8–26	8–22

^aFive biological replicates of 50 schizonts were counted for a total of 250 schizonts.