**Babesia divergens** builds a complex population structure composed of specific ratios of infected cells to ensure a prompt response to changing environmental conditions

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**Summary**

*Babesia* parasites cause a malaria-like febrile illness by infection of red blood cells (RBCs). Despite the growing importance of this tick-borne infection, its basic biology has been neglected. Using novel synchronization tools, the sequence of intra-erythrocytic events was followed from invasion through development and differentiation to egress. The dynamics of the parasite population were studied in culture, revealing for the first time, the complete array of morphological forms in a precursor–product relationship. Important chronological constants including *Babesia*’s highly unusual variable intra-erythrocytic life cycle, the life span of each population of infected cells and the time required for the genesis of the different parasite stages were elucidated. Importantly, the maintenance of specific ratios of the infected RBC populations was shown to be responsible for the parasites’ choice of developmental pathways, enabling swift responses to changing environmental conditions like availability of RBCs and nutrition. These results could impact the control of parasite proliferation and therefore disease.

**Introduction**

Life cycles of parasites are arrays of morphological forms, which emerge in an ordered sequence and are connected to one another in a precursor–product relationship. They give rise to cycles through constant transitioning from one form to another until interrupted by the host immune response or chemical compounds. During their life cycle, parasites undergo several cycles of extreme population growth within a brief span of time, which is critical for their continued transmission and a contributing factor for their pathogenesis. It is important to further our understanding of these processes in these organisms especially those that cause significant disease in both animals and man, as results from such studies can reveal novel targets for chemo-prophylaxis or immuno-prophylaxis.

Babesiosis is a zoonosis, a disease communicable from animals to man and an important blood-borne human parasitic infection (Vannier et al., 2015). Babesiosis has recently gained much attention because of its growing infection rate in humans by transfer from animal reservoirs and because it represents a potential threat to the blood supply, because asymptomatic infections in man are common and these can be life threatening in certain recipients (Lobo et al., 2013; Castro et al., 2014; Schmidt et al., 2014). The two major species of *Babesia* that cause human disease are *Babesia microti*, found primarily in the USA and *Babesia divergens* that is seen mainly in Europe (Gray et al., 2010; Hildebrandt et al., 2013; Vannier et al., 2015). Studies have focused on the latter because of the ease of its cultivation in human cells in vitro and the high parasitemias afforded by the cultures, which mimic those seen in human infections (Montero et al., 2006; Malandrin et al., 2009; Lobo et al., 2012; Tonkin et al., 2013; Cuesta et al., 2014; Jackson et al., 2014; Repnik et al., 2015). However, studies on its biology have been hampered by the lack of both appropriate technology and knowledge relating to its biological cycles. This contrasts with other Apicomplexan parasites like *Plasmodium* and *Toxoplasma*, which have been the focus of varied cell biology and molecular investigations (Francia and Striepen, 2014; Walker et al., 2014; Boucher and Bosch, 2015; Cheeseman and Weitzman, 2015). Of particular interest is the fact that *Plasmodium falciparum* and *B. divergens*...
infect the same cell, the human red blood cell (RBC). Pathogenesis of both malaria and babesiosis is intrinsically tied to parasite replication within RBCs followed by their lytic destruction, leading to anaemia and organ damage (Gray et al., 2010; Hildebrandt et al., 2013; Vannier et al., 2015). The proliferative cycles of these parasites differ substantially from their host and should therefore be exploited to ensure a steady pipeline of novel anti-parasitic treatments. To fulfil this promise, the unique structural and molecular features of parasite proliferation and development and differentiation within the host RBC must be understood.

In order to obtain a comprehensive understanding of the developmental cycles of *B. divergens* within the RBC, we undertook a systematic assessment of all developmental events starting from synchronized parasite invasion of the RBC. Earlier knowledge of the parasite was limited to a description of the three most frequently observed intra-erythrocytic (IE) life forms, which were thought to divide and egress out of the RBC in an IE cycle (IEC) of 8 h (Valentin et al., 1991). A recent paper shed more light on some aspects of the developmental cycle of the parasite, but because the studies were not carried out on synchronized parasites, sequential development together with accurate chronological parameters was not reported in that study (Rossouw et al., 2015). In this paper, we present a complete temporal resolution of the events in the *B. divergens*-infected RBC (iRBC) encompassing invasion, the various pathways of IE development available to the parasite and finally its egress from the RBC with the release of merozoites that initiate new cycles of infection. Apart from a detailed description of the cytological events related to parasite development and differentiation, our results further offer potential parasite imposed controls that drive this complex differentiation process.

**Results**

*Synchronized parasites cultures were achieved by purification of large number of viable-cellular merozoites and subsequent use in red blood cell invasion*

Large numbers of viable free merozoites were isolated by minimizing the stress to the parasites during experimental manipulation. The preparations were carefully examined by microscopy (Fig. 1A–C) and flow cytometry to ensure the absence of intact RBCs (Fig. 1D). In addition, the viability of the extracellular merozoites after isolation was assessed by evaluating their mitochondrial membrane potential (Fig. 1E) and examining the retention of overall morphology of the parasites by Giemsa staining (Fig. 1C). Of the free merozoites, 98% presented a high mitochondrial membrane potential (Fig. 1E), and all visible structural features were preserved after the manipulation. Thus, the overall viability of the free merozoites in suspension was high and free of intact RBCs (Fig. 1C).

*B. divergens* merozoites were viable for extended periods of time in vitro (up to 1 h). Therefore, in order to achieve tightly synchronized cultures, we limited the time of contact between the free merozoites and the RBCs to 5 min and followed by immediate washing to remove merozoites that had not yet invaded. This ensured that all parasites were synchronized within the tight 5 min developmental window. This short time of contact between host and parasite cells yielded tightly synchronized cultures (Fig. S1) with parasitemias ranging between 1% and 2%. In other experiments, when invasion was allowed for a longer period of time, parasitemias of ~10% could be obtained, albeit in not so tightly synchronized patterns. At 5 min post-invasion, no double invasion was observed, suggesting that the invasion of the RBC by multiple parasites was insignificant for the initial 48 h of synchronized growth.

**Growth pattern of parasite can be divided into two phases**

Tightly synchronized cultures obtained earlier were followed for 48 h by withdrawing aliquots from the cultures hourly and evaluated by Giemsa staining. Figure 2 shows that *B. divergens* cultures exhibited a two-phase developmental curve. The initial phase of culture occurred during the first 24 h post-invasion of synchronized culture initiation, when the parasitemia was seen to fluctuate between 1% and 2%. This was followed by the growth phase, from 24 to 48 h where the population density increases rapidly over time (2% to 5% in the period of 24–36 h; 5% to 8% in the period of 37–48 h), with a final parasitemia of ~7–8% recorded at the end of the 48 h period.

The initial phase (0–24 h) was shown to be highly dynamic in terms of variation of IE developmental forms but stable in terms of parasitemia (mean 1.5% ± 0.33). After 24 h, the culture shifted to growth phase and is characterized by both IE parasite development and events of new invasion, the latter being responsible for the increase in parasitemia between 24 and 48 h (mean 4.4% ± 1.9) (Fig. 2). The major cellular events that underlie these two phases of population growth are described in detail in the succeeding text.

*B. divergens* exhibits seven morphological stages of development

In asynchronous cultures, *B. divergens* reveals heterogeneity in both numbers and stages of IE forms. RBCs infected with single parasites coexist with others bearing multiple parasites that may not necessarily be in the same stage. By exploiting the synchronized cultures, we were able to identify the parasite developmental stages and to characterize the sequential transformation of one
stage to the next, defining the strict chronological order and timing of each event, beginning with merozoite invasion. In total, seven parasite stages could be identified, the three traditional stages (ring or pyriform; paired figure or figure "8" and the tetrad or Maltese Cross) plus double trophozoites (two sister cells), double paired figures, four trophozoites (four detached sister cells) and multiple parasites (more than four parasites per iRBC). The frequency distribution of all these stages as a function of time in culture is shown in Fig. 3A–G. It is worth noting, insofar as information on the cellular differentiation of *B. davenport* within the RBC is lacking, the term ‘trophozoite stage’ (single or multiple trophozoites) was used to generally refer to all intracellular parasitic cell(s), not attached to other parasitic cells, because, not possessing information about organelle differentiation, it is difficult to precisely discriminate among the various stages on the basis of Giemsa morphology. So, as shown in Fig. 3A, ‘single trophozoite’ refers to a single parasite either newly invaded or a trophozoite at feeding phase or dividing single cell. Similar terminology was used for double and quadruple trophozoites referring to two or four isolated intracellular forms that share the same host cytoplasm with sister cells. The electron micrographs confirm the viability and overall preservation of morphology of the parasites when present singly, in pairs or as multiple parasites within the host RBC (Fig. 3H–J).
The initial phase is the first 24 h of culture growth characterized by small variations in parasitemia. The growth phase, from 24 h post-invasion, shows pronounced increase in the population density over time as measured by parasitemia. Similar growth pattern is also seen in Fig. S2A. Parasitemia data are collected hourly from Giemsa slides, from 5 min to 48 h.

Chronology of morphological development in the infected red blood cell

As a testament to the synchronization of the cultures, only single trophozoites (100% of iRBCs) were observed in culture beginning from 5 min post-invasion to about 3 h post-invasion. After this, the percentage of single parasites steadily decreased reaching ~21% of iRBCs (the lowest frequency of single parasites seen in culture) at the 22 h time point (Fig. 3A). Conversely, iRBCs harbouring paired parasites gradually increased over time, reaching their peak frequency (53% of iRBCs) at 9–10 h post-invasion (Fig. 3B). Similar progression patterns for all other morphological stages were identified, with the paired figure stage followed by double trophozoites, which peaked at 14–15 h with ~18% of parasites in this stage (Fig. 3C); double paired figures next peaked at 16 h with 16% of parasites in this stage (Fig. 3D); followed by Maltese Cross stage seen to peak at 16–19 h with ~20% of parasites in this stage (Fig. 3E); and four trophozoites peaked at 21–25 h post-invasion having a frequency of 22–32%, (Fig. 3E). Finally, RBCs hosting multiple IE parasites could be seen at 29–35 h with 14–18% of parasites in this stage (Fig. 3G).

Comparing the highest to the lowest frequencies observed through the first 24 h cycle, we could calculate the ratio of parasites entering further development. Thus, 80% of single trophozoites progressed further developmentally, but a minimum proportion of ~20% remained throughout the cycle as single cells (100% after invasion and 21% as the ‘minimum’ frequency at 22 h) (Fig. 3A). In the same way, 80% of the paired figures went on to the next step of development, but 20% of all iRBC remained as paired figures (Fig. 3B). Unlike the single trophozoites, paired figures had two developmental options: to form double trophozoites or a Maltese Cross. As the maximum frequencies of both stages were very similar (15 h, 18% double trophozoites; 19 h, 21% Maltese Crosses) (Fig. 3C and E), we can infer that roughly half of the paired figures developed into double trophozoites, while the other half developed into Maltese Crosses. The Maltese Cross had a frequency maximum of 21% of all iRBCs in culture, but almost all of these (95%) gave rise to four trophozoites observed at a frequency of 32% of iRBCs at 24 h post-invasion. At 27 h post-invasion, only 1% of all iRBC were maintained as Maltese Crosses (Fig. 3E and F).

Of note, when the maximum percentage of four trophozoites was observed in culture at 24 h post-invasion (Fig. 3F), all parasite stages had already appeared, peaked and diminished in numbers in culture (exception for RBCs with multiple parasites, which appeared before 24 h but reached peak frequency later) (Fig. 3A–F). Thus, 24 h should be considered as the length of the IEC of the population as a whole. Furthermore, this peak of four trophozoites coincided with what we classify as the terminal point of the initial phase of the synchronized culture and the initiation of the growth phase of the population growth cycle (Fig. 2), whose onset is characterized by the rapid increase in parasitemia, resulting in the doubling and tripling of parasitemias. This rise in parasitemia can come about only with a concomitant rise in host cell invasion, and thus, the appearance of the four trophozoites stage, whether originating from Maltese Cross or from the double paired figures, is the developmental milestone for the end of a single-regimented developmental cycle of the parasite and defines 24 h as the duration of one complete IEC of *B. divergens*. This is confirmed by the progressive increase again in the frequency of single trophozoites seen in the RBC (after 24 h), signalling the second cycle of development, which was monitored between 24 and 48 h (Fig. 3A). During this second cycle of development, the peaks of each developmental stage were not as defined as the peaks during the first cycle because of the heterogeneity established by the first 24 h cycle.

Thus, using these synchronized parasites, it was possible to trace the developmental pathway and propose a model of *B. divergens* morphogenesis (Fig. 4) where parasites that have newly invaded RBCs (step 1) follow the pathway giving rise to paired figures (step 2). The latter in turn yields double trophozoites (step 3) or follows the typical development pathway forming Maltese Crosses (step 4). The double trophozoites (step 3) give rise to double paired figures that, in turn, give rise to four
Fig. 3. *B. divergens* morphological stages appear in a sequential and chronological order. (A–G) The frequency distribution of
A. single parasites as dots (immediately after invasion) or single trophozoites (throughout culture growth);
B. Paired figure;
C. Double trophozoites;
D. Double paired figures;
E. Maltese Cross;
F. Quadruple trophozoites; and
G. Multiple IE parasites.
Data are collected hourly from Giemsa slides, from 5 min to 48 h (light microscopy).
H–J. Electron micrographs demonstrating structural intactness of the parasites at
(H) single stage;
(I) double stage; and
(J) multiples trophozoites stages.

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trophozoites (step 3A) or double Maltese Crosses (step 3B). Single or double Maltese Crosses (steps 3B and 4) yield trophozoites (steps 3D and 4A) or follow additional developmental steps resulting in the accumulation of more than eight merozoites within the same iRBC (steps 3D and E, and 4B–E). These multiple parasites within the same iRBC (>4 IE parasites) are seen only at the later hours post-invasion and at high parasitemia (growth phase) and are limited to 10–12 parasites per iRBC but do not reach frequencies greater than 10% after their onset (Fig. 3G). Alternative morphological combinations are transient, representing intermediate stages interspersed among the seven basic stages and are generated from asynchronous proliferation among sister cells. Heterogeneity in this developmental model brings stability to the culture and is achieved by different populations of cells that follow distinct development pathways generating morphological diversity.

Parasite egress occurs first at 5 h post-invasion. Invasion occurs at regularly timed intervals during development as monitored by the periodic increase in parasitemia seen in our hourly monitored cultures. This was mirrored in the increase in frequency of trophozoites (two or more) observed over time. Invasion and egress can be considered as two sides of a coin, new invasion cannot occur unless egress has taken place to release the merozoites. While a reduction in parasitemia in a closely monitored culture would be the first indication of egress, this is difficult to establish as new invasion would rapidly restore parasitemia. Thus, an increase in the number of infected cells would indicate parasite egress that was followed by successful invasion. When assessed by light microscopy, the first decrease in parasitemia occurred between 4 and 5 h post-invasion (Fig. 2). A reduction of 24% in parasitemia from 1.4% at 4 h (Z-score: –0.52) down to 1% at 5 h (Z-score: –1.51) was observed. After
this time point, the parasitemia climbed at 7 h (a 64% increase in comparison with that at 5 h) (1.5% at 6 h, Z-score: −0.24; and 1.7% at 7 h, Z-score: 0.52), signalling that new invasive events had occurred (Z-scores related to 24 h mean 1.5% ± 0.33). This pattern was seen in multiple experiments as shown in Fig. S2A (Giemsa data) where between 2 and 4 h, the parasitemia dropped ~10% (1.6% at 2 h and 1.5% at 4 h, Z-score: −1.07) (5 h not sampled) at egress and increased ~88%, up to 2.8% at 6 h (in comparison with 4 h at 1.5%; Z-score: 1.35), when newly released merozoites invade RBCs (Z-scores related to 24 h mean 2.1% ± 0.54). Confirmation for this timing of egress was sought by examining the parasite morphology at these time points. The frequency of double trophozoites (not present before) increased from 0% to 3% of iRBCs between 3 and 4 h (Giemsa image, Fig. S1D; frequency of the double trophozoites stage compared with all iRBCs, Fig. 3C). Close examination of the morphology of the parasites shows that the presence of double (Giemsa, Fig. S1D; frequency of the double trophozoites, Fig. 3C) or quadruple trophozoites (frequency of the quadruple trophozoites, Fig. 3F) is a good indicator that a new cycle of invasion–development–proliferation will begin. Both the reduction in parasitemia and the presence of double trophozoites in culture indicate that the first event of egress occurs at 5 h post-invasion (based on the 5 h data as the shortest time interval between 4 and 6 h from Fig. 2). Thus, 5 h can be considered the smallest amount of time needed for one IEC.

DNA content analysis in combination with light microscopy confers a powerful platform to inform and describe the complex heterogeneity seen in the cultures

Flow cytometry was used together with microscopy in a second series of experiments on synchronized parasites to evaluate dynamics of parasite differentiation and proliferation together with morphogenesis. Parasitemia measured over 48 h using both fluorescence-activated cell sorting (FACS) and Giemsa (Fig. S2A), which clearly demonstrates that both analyses reveal comparable profiles of numbers of iRBCs in culture. The correlation between the parasitemia obtained from microscopy and FACS (Fig. S2A) was 0.98 (P < 0.001), and the correlation between the parasitemia results from both independent experiments (Giemsa curves: Fig. 2 and S2A) was 0.92 (P < 0.0001).

Based on the gating protocol presented in Fig. S2B, six populations of iRBC were identified as a function of their number of IE parasites/genome number (IE parasite load). When the FACS protocol was applied to a synchronized parasite culture initiated as described earlier, along with Giemsa staining and microscopy, the first iRBC population seen 5 min post-invasion was identified as 1 N population by assuming that 1 N refers to one genome copy corresponding to one haploid parasite within a newly invaded RBC. The other five iRBC populations were identified as 2 N-iRBC (two genome copies similar to two-cell pattern); 4 N-iRBC (four genome copies; similar to four-cell pattern); and three other >4 N-iRBC populations with more than four genome copies (>4Na-iRBC, >4Nb-iRBC and >4Nc-iRBC populations; similar to multiple infection patterns). We show the overlay of the frequency distributions obtained with both protocols in Fig. 5A–D. While both microscopy and FACS methods yielded comparable numbers of iRBCs (parasitemia), DNA content analysis did not discriminate between parasite morphology and genome number. Thus, iRBCs

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The time required for morphogenesis after DNA replication is shorter for two-cell stage parasites than four-cell stage parasites

To obtain chronological information required between DNA replication and the appearance of each stage (morphological differentiation of parasites), we analysed the interval between the initial time point for DNA replication (transitions from 1 N to 2 N termed as 2N-replication phase represented by 2 N-iRBC population and from 2 N to 4 N termed as 4 N-replication phase represented by 4 N-iRBC population) and for the onset of appearance of the morphological stages (zero time for each activity). Following the sequence of events through time plotted on the x-axis in Fig. 5B, the 2 N replication phase started between 1 and 2 h post-invasion when the frequency of 2 N-iRBCs began to increase (6% baseline to 9% at 2 h). There was no DNA replication activity observed before 1 h, and this was consistent with Giemsa-stained evidence of only single trophozoites being present in the culture at this point. The time period between 2 and 4 h was characterized by the appearance of paired figures (Giemsa) and the time period between 4 and 6 h by the onset of double trophozoites (Giemsa). From this observation, we can calculate that at the 2 N replication phase, there was a 2 h time interval between DNA replication (taking place between 1 and 2 h post-invasion) and the appearance of the paired figures (first seen between 2 and 4 h post-invasion). An additional 2 h was required for the appearance of double trophozoites (first seen between 4 and 6 h post-invasion). Moreover, at some time points, the fluctuation in the frequency of paired figures seems to be inversely proportional to the frequency of double trophozoites (black arrows in Fig. 5B), an indication that the two trophozoite stages was in fact derived from the paired figure stage.

The time period elapsing between the 4 N-replication phase and the appearance of four-cell stages in the iRBC (double paired/Maltese Cross/four trophozoites) was similarly investigated. Little DNA replication activity was observed between 2 and 10 h (1% as baseline frequency of 4 N-iRBC ranging 3–6% between 2 and 10 h) (Fig. 5C).

The frequency of 4 N-iRBCs slowly but steadily increased between 10 and 18 h post-invasion, reaching a maximum of 38% at 18 h. This increase in number of 4 N-iRBCs was reflected in the Giemsa stained smear microscopy, which revealed the appearance of double paired figures mainly between 14 and 16 h (5% at 16 h; peaking ~14% at 22 h) followed by Maltese Cross at 16–18 h (6% at 18 h; peaking 18% at 22 h) and leading to the appearance of four trophozoites at 20–22 h for the first time in culture (4% at 22 h; peaking 12% at 24 h). In comparison with two-cell stages (trophozoites differentiating into paired figures and then into double trophozoites) (Fig. 5B), the morphogenesis of four-cell stages (single paired figure differentiating into Maltese Cross or double trophozoites differentiating into double paired figures; and then into four trophozoites) (Fig. 5C) took longer, because double paired figures (between 14 and 16 h) appeared 4 h after 4 N-replication phase had started (between 10 and 12 h); and Maltese Cross appeared 6 h (between 16 and 18 h) after the start of the 4 N-replication phase. Thus, the morphogenesis of two-cell stages required in total 4 h to generate double trophozoites (Fig. 5B), while the morphogenesis of four-cell stages needed 8 h to generate four trophozoites (Fig. 5C). It is important to stress that Maltese Cross parasites are not the progeny of double paired figures but instead result from two rounds of cell division with sister cells remaining attached after mitosis.

In the growth phase of the culture (24–48 h), cultures gained complexity and heterogeneity progressively through the parasite development. This made difficult the identification of the relationships between morphological transitions and replication cycles. However, the relationship between DNA composition and morphological stages was evident throughout the time period followed, because regular fluctuations in stage frequency were observed even after 24 h. Additional heterogeneity in post-24 h invasion cultures come from the >4 N-iRBC populations (Fig. 5D), whose frequency appears to be inversely proportional to the number of IE parasites they host (IE parasite load). In comparison with 4 N-iRBC, all three >4 N-iRBC populations showed a low frequency at initial phase (0–1%; >4 N-iRBC: 4 N-iRBC 11–38%), but their frequency increased to ~3% after 24 h of culture (Fig. 5D).

A single cycle defining the span of a specific infected red blood cell population in culture takes 18 h

To define the ‘life span’ of each morphogenetic form of the parasite, we analysed the frequency of the seven morphological stages. As shown in Fig. 6A, it was not possible to identify a constant time interval between stages. We thus turned to an analysis of the frequency of the six iRBC populations with different IE parasite loads to assess the relative time spans of each iRBC population instead of stages (Fig. 6B). Figure 6B presents an overall
superimposition of the frequency graphs of iRBC populations (with varying nuclear content) during the 48 h period as seen in synchronized cultures. This analysis permitted a definition of the time taken from the appearance of a specific iRBC population in culture, through its peak and subsequent decline and from there on to its second cycle in culture. This parameter has been termed ‘life span’ for that particular iRBC population. In the 48 h time period of analysis, we observed three peaks representing high frequency in culture for the 1 N-iRBCs and two peaks each for the 2 N-iRBCs and 4 N-iRBCs (Fig. 6B). In all frequency curves plotted in the graphs of Fig. 6B, the highest frequency was kept constant for 2 h, which resulted in a plateau formation present at the top of the peaks. These plateaus served as a reference to identify the related time points in the different curves of iRBC populations. As there was no temporal overlap among peaks, the sequential transformation from 1N-iRBC to 2N-iRBC and then to 4N-iRBC is clearly observed. As indicated by the upper bars in Fig. 6B, we can demarcate a regular 6 h time interval that occurs between sequential iRBC populations. Thus, 6 h defines the time distance between 1 N-iRBC and 2 N-iRBC (one asterisk: 24–30 h) (Fig. 6B); 2 N-iRBCs and 4 N-iRBCs (two asterisks: 30–36 h); and 4 N-iRBC and 1 N-iRBC (three asterisks: 18–24 h).

Having defined the interval between sequential categories of iRBCs loaded with specific populations (1 N, 2 N, 4 N and >4 N), we next characterized the time required for the completion of a cycle of specific iRBC populations exploiting two key time points in their frequency charts: the time point corresponding to their lowest frequency before formation of the peak and the time point corresponding to just after dissolution of the peak. As indicated in Fig. 6B by the lower bars, 18 h was the time required to complete one cycle of each of the iRBC populations found in culture.

Eighteen hours was also the time period needed for one round of transformation from 1 N to 2 N; 2 N to 4 N; and 4 N back to 1 N to take place (6 h for each step). Thus, using this analysis of frequency of each iRBC population, we could define the first cycle for 1 N-iRBCs as taking place from 0–18 h, for 2 N-iRBCs from 20–36 h and for 4 N-iRBCs from 24–30 h. Again, in the second cyclic rounds of parasite development, 1 N-iRBCs were seen from 18–36 h and 2 N-iRBCs from 20–38 h.

Comparing the dynamics of the three main iRBC populations through the 48 h monitored period, an interesting feature of the frequencies was presented. At every 18 h of synchronized growth, the proportion of 1 N-iRBC, 2 N-iRBC and 4 N-iRBC populations were similar (~30% each) (Fig. 6B). At 18 h and 36 h time periods, while 1 N-iRBCs and 2 N-iRBCs were at their lower frequency (~30%), 4 N-iRBCs were at their highest frequency (~30%), at both time points. This condition, where different iRBC populations shared similar representations in culture, remarkably coincided with the 18 h life cycle of the iRBC populations and the end of the 1 N-iRBC cycle. In 48 h, we recorded three cycles for 1 N-iRBC population (the last one not completely seen) being separated by the two time points (18 h and 36 h). As seen before, the increase in 1 N-iRBCs frequency (~22 h, Figs 5A and 6B) preceded the population density gain (Fig. 2). We thus hypothesized that this maintenance of relative proportions of the various iRBC populations is an additional mechanism that B. divergens uses to control the population density. These 18 h time points appear to function as ‘check points’ that not only signal the ending of a 1 N-iRBC cycle but also appear to control the initiation of new 1 N-iRBC IE parasite cycles by increasing events of invasion. To test this hypothesis, we evaluated asynchronous cultures under different conditions for parasite replication.

Fig. 6. B. divergens proliferative cycle defines the life span of iRBC populations.
A. Comparison among the frequencies of all seven morphological stages light microscopy data. (G-Giemsa)
B. Comparison among the six iRBC populations using different IE parasite load: 1 N-iRBC; 2 N-iRBC; 4 N-iRBC; >4 N-iRBCa, >4 N-iRBCb and >4 N-iRBCc populations.
Time points 18 h and 36 h (black arrows) refer to check points for parasite population to make developmental choices. Time points: 5 min, 1 h and 2 h and after that every 2 h. FACS data.
B. divergens exerts similar controls in asynchronous and synchronous cultures

Eight independent asynchronous cultures of varying parasitemia (represented as an average of three independent cultures for each parasitemia, set from 0.9% to 48%) were characterized for their DNA content (FACS) as a function of parasite density (Fig. 7A). Except for the 2 N-iRBC population whose frequency was stable among all asynchronous cultures (~38%), the frequency of other iRBC populations varied, proportionately increasing (4 N-iRBC and >4Na-iRBC, >4Nb-iRBC, >4Nc-iRBC populations) or decreasing (1 N-iRBC population) with a rise in population density as measured by parasitemia (Fig. 7A). As shown before in synchronous cultures (Fig. 5D), the appearance and increased frequency of iRBC carrying high IE parasite loads was related to high parasitemia (in decreasing order: >4Na, >4Nb and >4Nc). The frequency of iRBC with equal to or greater than 4 N parasite load among these eight cultures (1–48% parasitemia) increased from 19% to 25% for 4 N-iRBC; from 3% to 9% for >4Na-iRBC; from 2% to 7% for >4Nb-iRBC; and from 0% to 2% for >4Nc-iRBC populations, whereas the frequency of 1 N-iRBC population decreased from 38% to 17% (correlation between the frequencies of 1 N-iRBC and all iRBC populations with equal or greater than 4 N DNA content was −0.98, P < 0.001). These results reveal that the fluctuation in proportions of the various iRBC populations is not exclusive to synchronous cultures and reinforces the idea that the parasite maintains 1 N-iRBC and 4 N/4N-iRBC populations as the variable populations. This corroborates our previous data showing that B. divergens culture is not static and, interestingly, shows an additional mechanism dictated by the availability of host cells that control the culture expansion by means of increasing the IE parasite load and slowing down the invasion process as parasitemias increase.

B. divergens controls population expansion based on the availability of host cells and nutrients

The rate of population growth, as monitored by an increase in parasitemia, also decreases as the parasite population reaches the stationary phase (50% parasitemia) (Cursino-Santos et al., unpublished data). We thus analysed the composition of the different parasite populations in response to these elevated parasitemias that could be translated as nutrient or host cell deprivation (Fig. 7B and C). We evaluated the frequency of the various iRBCs populations in two asynchronous cultures at high parasitemia (parasitemia average 46%; one at 43% and one at 49%) subjected to nutrient deprivation by not changing the culture medium for about 10 h. Under
such limitation conditions, the culture at the parasitemia of 46% [zero time (ZT) in Fig. 7B and C] showed frequencies of each iRBC populations (1 N-iRBC, 2 N-iRBC and 4 N-iRBC) at similar proportions, ~30%. When fresh medium was added (Fig. 7B) or when new cultures were initiated from the high parasitemia cultures (initiated at 1% parasitemia) (Fig. 7C), the frequency profiles of the iRBC populations changed. When the supplement of nutrients was re-established (medium changed multiple times in 24 h), the parasitemia increased to 63% and the frequency profile that was assessed at 46% overall parasitemia in culture: 1 N, 30%; 2 N, 32%; 4 N, 28%; >4Na, 7%; >4Nb, 3% >4Nc, 0% changed to 63% overall parasitemia in culture with the following proportions of iRBCs: 1 N, 19%; 2 N, 34%; 4 N, 31%; >4Na, 8%; >4Nb, 5%; >4Nc, 4% (Fig. 7B). These results reinforce our hypothesis that B. divergens has an efficient control over parasite proliferation by switching the distribution of the three main iRBC populations, 1 N-iRBC, 2 N-iRBC and 4 N-iRBC from the ‘check point pattern’ (30:30:30) as previously observed in synchronized cultures at 18 h and 36 h (Fig. 6B) but induced here by nutrient decrease (availability of host cells but insufficient nutrients) to a ‘merozoite storage pattern’, where the egress/invagination was inhibited (low frequency of 1 N-iRBC, 20%) and higher frequencies of multiple infective units stored within the same iRBC (>4Na-iRBC, 8%; >4Nb-iRBC, 5%; >4Nc-iRBC, 4%) (Fig. 7B).

When new host cells were introduced into the density-stressed culture (subculture from ~50% parasitemia to 1% parasitemia) (Fig. 7C), the parasite promptly responded to the favourable condition by increasing the 1 N-iRBC population from 29% at zero time (46% parasitemia at ZT) to 32% at 30 min post-splitting (1% parasitemia at 30 min) to 37% at 24 h post-split (3% parasitemia at 24 h) (Fig. 7C). Furthermore, by comparing the distribution patterns of iRBC at zero time, 30 min and 20 h post-splitting, it is shown that the favourable condition for population growth (parasitemia increasing from 1% to 2%) was reflected in the changes in the frequency profiles of all iRBCs in culture (Fig. 7C), where positive signals for invasion and egress are seen by the increase of 1 N-iRBC population and the decrease of 4 N-iRBC population respectively (Fig. 7C). By the increase of 2 N-iRBC (from 32% at zero time to 39% after 24 h splitting), a clear balance between invasion, proliferation and parasite differentiation is maintained, dictated by the availability of host cells.

Discussion

The study of biological cycles in B. divergens has been hampered by the fact that synchronization of in vitro cultures was not feasible using methods successful for related Apicomplexans like P. falciparum (Lambros and Vanderberg, 1979; Russmann et al., 1982; Wahlgren et al., 1983; Kim, 2004) Theileria (Sugimoto et al., 1991) and Babesia bovis (Rodriguez et al., 1986). Thus, methods based on density differences like Percoll centrifugation or osmotic lysis of mature malaria stages (Lambros and Vanderberg, 1979; Russmann et al., 1982; Wahlgren et al., 1983) have not yielded synchronous cultures. Other methods that isolate free merozoites spontaneously released in B. divergens culture (Montero et al., 2006) and electroporation of iRBC (Franssen et al., 2003; Sun et al., 2011) result in low invasion rates in our hands. By avoiding low temperatures (Cursino-Santos et al., 2014) and multiple centrifugations, and using a novel method to release infectious merozoites to infect RBCs, we were able to obtain highly synchronized parasite populations to exploit for a detailed chronological characterization of the IEC of B. divergens. Unlike the sorting method of asynchronous populations of B. divergens iRBC (Rossouw et al., 2015), our synchronization yields initial homogenous iRBC populations. B. divergens merozoites are capable of infecting RBCs even an hour after isolation and purification unlike P. falciparum merozoites, which are viable for a few minutes (Gilson and Crabb, 2009). This extended period of viability dictated a short time of contact between RBC and parasite to achieve tightly synchronized parasite populations, which served as an optimal substrate to define the dynamics of parasite population growth and differentiation.

It is important to emphasize that despite the complementarity of microscopy and flow cytometry methods, they are measuring different parameters, and the absolute comparison of numbers is possible only when the number of intracellular parasite cells is the same as their genome number. Discrepancy between microscopy and flow data is expected for some intervals of time in the various phases of parasites development. For example, at the 8 h time point, the majority of parasites in culture are present as paired figures, which were recently converted to 2 N and two-cell stage (DNA content matches with two-cell stage frequency). However, the interval between 8 and 16 h is a very dynamic period for the culture with several events taking place. The fluctuation in frequency of paired figures is a consequence of the completion of cell division for some paired figures with the consequent appearance of double trophozoites, which in turn, can stay as double rings (2 N), or egress, or can even follow the development. In this case, cells going into the second round of cell proliferation (two-stage cells developing in four-stage cells) have doubled their DNA content (being measured by FACS as a 4 N-iRBC population) despite the fact that by Giemsa staining, they still appear as a single paired figure and double trophozoites or single paired figure. For this reason, between two proliferative cycles, when parasite cells are between the interphase and cytokinesis
phases of the cell cycle, a deviation is expected between the measurement of DNA content and cell number. So, parasite enumeration by microscopy will identify iRBCs bearing two-stage, cells and this same population will be shown to be 4N-iRBCs by FACS, if the parasites are at the proliferative stage. This is confirmed by the comparison between Fig. 5B and C. Between 12 and 18 h, iRBCs with 2N parasite DNA content drops (Fig. 5B), and in contrast, iRBCs with 4N parasite DNA content increases (Fig. 5C). However, the parasites that have not concluded their mitoses yet are still recorded as single paired figure or double trophozoites (Fig. 5B) and this makes the sum of their percentages higher than that of the 2N-iRBCs. So while microscopy and flow cytometry can be used in parallel to complement each other, strict attention must be paid to interpretation of data to reveal the biological cycle.

Different terminologies have been used to describe the morphological variation of Babesia species as seen by light microscopy, with some studies referring to parasite forms (ring, piroplasm, anaplasmoid, amoeboid and tetrad) (Kjemtrup and Conrad, 2000, 2006) and others as parasite stages (ring, paired figure and Maltese Cross) (Gray et al., 2010). This inconsistency in terminology comes from a lack of knowledge of the cell biology of this understudied species. We have interpreted the ‘trophozoite’ term to refer to all unattached cells present within an RBC irrespective of their cellular differentiation (seen by Giemsa as post-invasion forms as ‘dots’ immediately after invasion and newly formed ring or when further along the developmental pathway but still presenting as a ‘ring’). The same limitation exists for double, quadruple or multiple unattached parasites sharing the same host cell. For this reason, we generically call all detached cells as trophozoites, and these can vary in number from 1 to 12 parasite cells. It is only after further structural and gene expression studies that a more exact and specific terminology can be adopted to characterize different parasite forms of the complex population of B. divergens.

Our results have clearly identified seven distinct IE parasite stages in contrast to the three that have been previously discussed. Additionally, we were able to trace the chronological order and sequence of each of these seven morphologically distinct stages, leading to the development and differentiation model illustrated in Figs 3 and 2. This pattern was strictly chronological, with each morphological form following its developmental cycle. The appearance of a new stage in culture was always preceded by a reduction in frequency of the previous stage. Thus, B. divergens development in the RBC is a tightly coordinated sequence of morphological transformations. We show that this morphogenesis is strictly related to the proliferative activity, which in turn is related to the population density. Independent of the parasitemia and of the synchronicity of the culture, we found that 6h (interval between the maximum frequencies of two populations 1N-iRBC, 2N-iRBC or 4N-iRBC; Fig. 6B) is needed for the culture to double the IE parasite load (DNA content) (defined in this study as proliferative cycle). As there was no complete loss of any developmental stage (all cellular structures of parasites are preserved in all stages, Fig. 3H–J), the cultures lost synchronicity and gained complexity and heterogeneity, not through a random choice of developmental options, but instead through developmental controls that B. divergens exercises as a means of balancing growth and new cell invasion.

Merozoites are the only parasite form that can egress to allow new invasion resulting in rise in parasitemia. However, these infective units can arise from multiple parasite reservoirs like the paired figure or the Maltese Cross or two paired figures or the >4N populations. Depending on the favourable conditions for population expansion, these infective units are released to initiate new infective cycles. If there is an urgent need for culture expansion, two merozoites will be released from the RBC, diverting the parasite away from differentiation of double trophozoites into double paired figures or paired figures into Maltese Cross formation. Earlier reports on another Babesia species, B. microti, suggested that the appearance of the Maltese Cross is related to high infectivity (Yokoyama et al., 2003). This is in agreement with our analysis of the infective potential of these stages to release higher numbers of merozoites. Previous studies defined an 8h IE life cycle for B. divergens although no clear rationale for this important chronological hallmark had been laid out (Valentin et al., 1991). More recently, a 4h life cycle (Rossouw et al., 2015) was proposed for the IEC of B. divergens, based on the first events of egress. While, our data agree with the 4–5h as representation of the shortest IEC of the parasite, it also points to a more complex definition of the IEC of the parasite population as a whole and is the time required for a manifestation of all seven morphogenetic forms of the parasite together with clear periods of parasite egress (reflected in decrease in parasitemia) followed by new host cell invasion (reflected by a concomitant increase in parasitemia). As summarized in Fig. 8, 24 h is the time needed for the population to complete these events, 6h between single and double trophozoites seen by completed cytokinesis with two-stage cells; and 24h for the complete morphogenesis cycle, from single to quadruple trophozoites, peaking at 24 h and multiple parasites, where the diversity of parasite forms is a consequence of incomplete cytokinesis; or the other way around, 4 h from single trophozoite to paired figure plus 16 h from paired figure to Maltese Cross and plus extra 4 h from Maltese Cross to quadruple trophozoites (Figs 5, 6A and 8). Support for this estimate comes...
Populations seen in culture and an additional 6 h is needed from the fact that 18 h defines the life span of each iRBC. Events take place at 5 h post-invasion. The first egress when only double trophozoites egress, and higher rates of invasion (Fig. 8). Low invasion rates are seen rates depending on the phase of culture growth and the output of free merozoites after egress (blue arrows). Low invasion occurs with the transformation of single trophozoites into paired figures and morphological differentiation maintaining 18 h as the life span of invasion (red arrows) occur through this entire 24 h cycle at different locations. The sequential transformation in the different populations has already reached their highest frequency peaks in culture, and with an additional 6 h (grey clock), the morphogenesis is completed with quadruple trophozoites reaching the highest frequency in culture (24 h, dark grey gradient). The morphogenesis cycle (external pink clock with iRBCs), with the culture at initial phase with low initial parasitemia, starts between 2 and 4 h post-invasion with the transformation of single trophozoites into paired figures and then into double trophozoites between 4 and 6 h (total 6 h from single to double trophozoites). The sequential transformation in the different stages ends with the quadruple trophozoites and multiple intracellular parasites at 24 h post-invasion (appearance of double paired figure and Maltese Cross between 14 and 18 h; and appearance of quadruples trophozoites appearance between 20 and 22 h; total), from where the parasitemia exponentially increases. Events of invasion (red arrows) occur through this entire 24 h cycle at different rates depending on the phase of culture growth and the output of free merozoites after egress (blue arrows). Low invasion rates are seen when only double trophozoites egress, and higher rates of invasion occur with the releasing of quadruple trophozoites. The first egress events take place at 5 h post-invasion.

Using the synchronized parasite cultures, important chronological constants that govern the parasite lifecycle could thus be calculated allowing precise definitions of the biological clocks of B. divergens (Fig. 8).

B. divergens builds an appropriate mix of subpopulations of iRBCs (1N-iRBC; 2N-iRBC; and ≥4 N-iRBC populations) that provides for the parasite population high flexibility in terms of morphogenetic options based on the proliferative needs of the population, which in turn is dictated by availability of host cells and nutrients. It appears that a form of control may be taking place in B. divergens population in which, at high parasitemia, options to prolong the IEC are taken, whereas at lower parasitemia, exiting the RBC is chosen to invade new cells and increase population numbers. This developmental choice is unique for Babesia compared with other Apicomplexans like P. falciparum and Toxoplasma gondii, which have precisely timed IE cycles. P. falciparum typically spends 48 h in each erythrocytic cycle, while tachyzoite of T. gondii has a 6 h growth and replication period inside the host cell before egress (Nishi et al., 2008). Gametocytogenesis is the only other available developmental choice for malaria, but no other asexual developmental options exist for P. falciparum (Lobo and Kumar, 1998).

Work reported in this paper, using synchronized parasite populations for the first time, clearly reveals not only the sequential progression of the seven morphological forms of B. divergens in culture but also the dynamics of parasite proliferation and differentiation that are maintained through controls that secure the constituent iRBC populations in strict ratios to enable rapid movement between new invasion events or further IE development and replication cycles. Strict temporal control of each parasite life cycle was also seen in the chronological constants outlined by our data. Notably, the underpinning mechanistic control in terms of molecular pathways responsible for these developmental choices remains to be elucidated and will be the focus of future studies. Outcomes of such research will enhance further understanding of the developmental biology of B. divergens and direct development of new therapeutics that can interrupt the lifecycle of this important human and veterinary pathogen.

**Experimental procedures**

**Ethics statement**

Human blood from healthy volunteer donors was used to culture B. divergens in vitro. The blood was de-identified and approved for use by the New York Blood Center Institutional Review Board (NYBC IRB). All blood donors gave informed written consent for use of their blood for research purposes.
**B. Divergens in vitro culture**

*Babesia divergens* (Bd Rouen 1986 strain) were maintained in human RBCs at 5% haematocrit in complete medium (RPMI 1640; Life Technologies Corporation, Carlsbad, CA; supplemented with 50 μg ml⁻¹ of hypoxanthine; Sigma; 0.24% (v/v) sodium bicarbonate; Life Technologies Corporation; and 10% human serum) under low oxygen atmosphere (5% O₂, 90% CO₂, 5% N₂) at 37°C, as previously described (Gorenflot et al., 1991). A+ RBCs were collected in 10% Citrate Phosphate Dextrose (CPD) and washed three times with incomplete RPMI 1640 medium for the complete plasma and white cells removal.

**Free merozoites isolation**

High concentration of viable free merozoites was isolated from 25 ml of unsynchronized cultures at high parasitemia (40%) based on a previously described protocol with modifications (Precigout et al., 1993; Montero et al., 2006). In order to minimize the stress to the parasites during experimental manipulation, all procedures were carried out at room temperature, and the frequency of centrifugation was minimized in all procedures involving live parasites, including the isolation of free merozoites and culture synchronization. Haematocrit of cultures was reduced to ~30% haematocrit by aspiration and filtered once with 5 μm and twice with 2 μm filters (Versapor® membranes) using 1mL syringe. Centrifugation (670 g; 2 min) was applied only after the last filtration. The supernatant (suspension of free merozoites) was used as inoculum of new cultures for parasite synchronization. All free merozoite preparations were evaluated for intact membranes) using 1 mL syringe. Centrifugation (670 g; 2 min) was applied only after the last filtration. The supernatant (suspension of free merozoites) was used as inoculum of new cultures for parasite synchronization. All free merozoite preparations were evaluated for intact membranes and washed three times with incomplete RPMI 1640 medium for the complete plasma and white cells removal.

**Culture synchronization**

Fresh cultures were seeded with purified merozoites suspension at 10% (v/v) of culture volume. After 5 min of incubation, the cultures were washed twice with warmed 37°C complete medium (670 g; 2 min) to reduce the late RBC invasion by free merozoites removal. Samples were collected at specific intervals of time: Every hour up to 48 h for the initial development characterization of *B. divergens* by light microscopy and every 2 h up to 24 h for the cellular proliferation analysis by FACS. Cells were obtained alternatively from three synchronized cultures prepared from a max culture (15 mL that received the inoculum) that was split into three flasks after the free merozoites removal in order to preserve the culture stability and homogeneity among the three flasks.

**Microscopy**

Light microscopy was used for the morphological analysis of parasites through development and for parasitemia evaluation. It was also used in parallel to flow cytometry to identify the cell populations related to the different parasites stages. Blood smears were fixed with methanol and stained with Giemsa (number of iRBCs in every 100 RBCs). A minimum of 2000 cells were counted per slide using a Nikon Eclipse E 600 microscope.

**Statistics**

Parasitemia was calculated by blood smears from cultures stained with Giemsa defined as the total number of iRBCs in every 100 RBCs not taking into consideration the number of parasites seen in a given cell. The statistics were calculated by Microsoft Excel (2007) or SAS software, version 9.4 2012 (SAS Institute Inc., Cary, NC, USA).

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**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

**Fig. 51.** Tightly synchronized cultures were obtained by limiting contact between RBC and purified merozoites.

(A-C) Synchronized differentiation from rings to Maltese Cross with the parasites reaching the same morphological stage of development at the same period of time: (A) Merozoites seen as minute dots at 5 min post invasion; (B) paired figures at ~8 h; (C) tetrad form or Maltese Cross at ~16 h. (D) Presence of double trophozoites at ~5 h and (E) multiple intra cellular parasites at ~24 h associated to events
of egress. (F) Culture heterogeneity at ~48h. Data collected hourly from Giemsa slides, from 5 min to 48 h. Light microscopy.

**Fig. S2.** FACS and Giemsa serve as complementary methods to describe the composition of B. divergens cultures.

A. Parasitemia data obtained from Flow Cytometry (orange curve) and light microscopy (blue curve, Giemsa stain) yield comparable results between both methods and between different experiments (see Giemsa data, Figure 2). In synchronized culture, a discreet variation in parasitemia is repeatedly observed between 2-6 h post invasion (1st events of egress followed by new events of invasion with consequent increase in parasitemia). Time points: 5 min, 1h and 2h and after that every 2 hours.

B. Bivariate distribution of cells from an asynchronous culture used for the gate settings. Plot showing cells double stained with Vybrant®DyeCycleTMGreen and BV421-GPA to discriminate between infected (iRBCs, red box) and uninfected RBC (uRBCs black box to the left size). Top: gates referring to all RBC (iRBCs and uRBCs). Six iRBC populations are identified by the differences in their DNA content or the number of intracellular parasite genomes (intra erythrocyte parasite load). (1N-iRBC): iRBCs with 1N parasite genome. (2N-iRBC): iRBCs with 2N parasite genome. (4N-iRBC): iRBCs with 4N parasite genome. (>4N-iRBCa, b, c) three different >4N-iRBC populations with increasing DNA content. On the bottom right side, the green population refers to free merozoites with 1N or more genomes at the time of their release from the lysis of RBC. On the bottom left side, debris or cell fragments. (WBC) white blood cells. All washed RBC used in culture were previously tested for WBC contamination. Events acquired by flow cytometry.