

Review

The Challenge of Quantifying Synchrony in Malaria Parasites

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Malaria infection is often accompanied by periodic fevers, triggered by synchronous cycles of parasite replication within the host. The degree of synchrony in parasite development influences the efficacy of drugs and immune defenses and is therefore relevant to host health and infectiousness. Synchrony is thought to vary over the course of infection and across different host–parasite genotype or species combinations, but the evolutionary significance – if any – of this diversity remains elusive. Standardized methods are lacking, but the most common metric for quantifying synchrony is the percentage of parasites in a particular developmental stage. We use a heuristic model to show that this metric is often unacceptably biased. Methodological challenges must be addressed to characterize diverse patterns of synchrony and their consequences for disease severity and spread.

Why Synchrony Matters

Periodic fevers have long been observed in human malaria infections (e.g., [1]), where regular spikes in temperature serve as an outward manifestation of synchronized cycles of parasite replication within the blood [2]. As parasites multiply within red blood cells (RBCs), they progress through developmental stages with distinct properties (see Figure 1 in Box 1); the extent to which the parasite population progresses through this cycle in unison is therefore likely to influence transmission success [3] and clinical outcomes [4]. **Synchrony** (see Glossary) has been alternately hypothesized to be beneficial for hosts (limiting debilitating symptoms to brief, regular intervals [2]), for parasites (enabling parasites to match circadian rhythms within their vertebrate hosts [5,6], or the insects that vector them [7–10]), for both (reducing over-depletion of host resources [3]), or for neither (reviewed in [11]). Understanding how synchrony impacts host health and malaria transmission represents an ongoing challenge.

The drivers of synchrony remain elusive. Not all infections are synchronous, and even synchronous infections of humans may not elicit periodic fevers [12]. Within groups of broadly similar hosts, different patterns of synchrony have been observed (e.g., asymptomatic children [12]; primary infections of neurosyphilitic patients [13]). Malaria infections can persist for many months (e.g., [14,15]), over which time the level of synchrony is thought to change. Classic literature posits that synchrony increases after the onset of symptoms as immune responses intensify, a process thought to occur more quickly in individuals previously exposed to malaria parasites [2]. Synchrony has also been hypothesized to erode when strains persist in the host for many cycles, as subtle differences accumulate in the timing of replication across the parasite population [16]. Synchrony is lost rapidly – within a few cycles of replication – when human malaria parasites (*Plasmodium falciparum*) are artificially cultured [17], suggesting that some aspect of host physiology maintains synchronized cycles of replication. Experiments with rodent malaria parasites (*Plasmodium chabaudi*) suggest that host feeding rhythms – especially circadian rhythms in glucose metabolism – play an important role in maintaining parasite

Highlights

Periodic fevers are the classic malaria symptom, generated by synchronous cycles of parasite development and multiplication within the blood.

Synchrony is not ubiquitous across, or even within, malaria infections, and the degree of synchrony is likely to alter transmission success and health outcomes.

Comparing the degree of synchrony across species, strains, and time points within infection can illuminate the proximate and evolutionary drivers of synchrony.

Standardized methods for comparing synchrony are lacking; only one existing method – estimating the percentage of parasites in a particular developmental stage – can be applied to both infections within the host and to artificial cultures of parasites.

Applied to the problem of estimating synchrony, stage percentages data suffer from multiple sources of bias, highlighting an urgent need for better approaches.

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Box 1. Parasite Success Is Stage-Dependent

The level of synchrony has the potential to alter infection outcomes since parasites interact with their environment in a stage-specific manner (Figure 1). Many drugs (including the current front-line drug artemisinin) are most effective at removing parasites during metabolically active stages [20], though drugs vary in their stage specificity [21,22]. An infection therefore may appear resistant to drug treatment – that is, drugs may initially fail to perturb parasite dynamics – merely because the parasite population is synchronized in a developmental stage that is relatively unaffected by drugs at the time of treatment [4]. Beyond drug treatment, many interactions have the potential to be both stage- and parasite density-dependent, including competition for RBCs [33], the propensity to trigger an immune response and the ability to overwhelm different components of immunity [55,82]. Thus, the extent of synchrony represents an important component of within-host ecology.

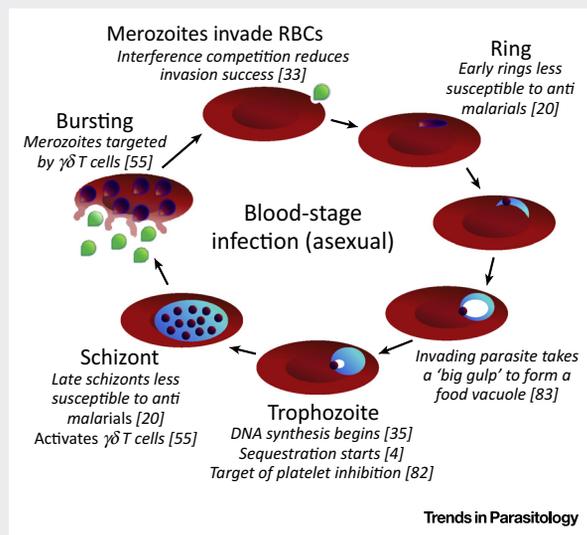


Figure 1. Stage-Specific Ecology. Parasite developmental stage influences competition for red blood cells (RBCs) to invade [33], susceptibility to antimalarial drugs [20], metabolic needs [35,83], propensity to sequester [4], and interactions with host defenses [55,82].

synchronization [5,6]. Whether hosts impose synchronous development on parasites via circadian rhythms in nutrient availability, or whether parasites synchronize development to coincide with those rhythms, is unclear [18], but two lines of evidence suggest that parasites themselves also influence the level of synchrony. First, parasite species infecting the same host species can exhibit different levels of synchrony (e.g., the human malaria species *P. falciparum* and *Plasmodium vivax* [2]; rodent malaria parasites infecting mice, reviewed in [11]). Second, cultured *P. falciparum* strains lose synchrony at different rates following artificial synchronization [19]. Further, synchrony appears to vary across parasite species, within parasite species, and within a single infection through time, and those patterns suggest intriguing hypotheses about the drivers of synchronized dynamics (reviewed in [11]). This observed variation should be useful for disentangling the proximate causes of synchrony, but standardized methods of quantifying synchrony are lacking.

Such methods are needed to understand evolutionary outcomes. Theory predicts that synchrony can enhance transmission success [3], but it is unknown whether there is heritable variation among parasites in patterns of synchrony, a prerequisite for evolution by natural selection. Thus, whether synchrony represents a parasite adaptation (or is even a trait that can evolve) remains an open question, and one with considerable applied significance. Antimalarial drugs tend to be most effective against particular developmental stages

Glossary

Artemisinin: a current front-line antimalarial drug with a half-life much shorter than the parasites' cycle length. Drug efficacy *in vitro* depends on developmental synchrony and the timing of drug treatment [58].

Cycle length: the period of time required for parasites to replicate within RBCs, that is, the duration of time from merozoite invasion to bursting.

Gametocyte: a specialized parasite stage capable of infecting mosquitoes; it cannot invade red blood cells.

Intraerythrocytic development: the process of parasite replication within an RBC following merozoite invasion.

Merozoite: a red-blood-cell-invasive parasite (a product of intraerythrocytic development).

Ring stage: the initial stage of intraerythrocytic development, comprising approximately half the cycle length [19,70].

Schizont: the final stage of intraerythrocytic development that terminates when the RBC bursts to release merozoites, representing about one quarter of the cycle length [19].

Sequestration: the process by which some parasite stages are retained in small blood vessels rather than circulating freely through the bloodstream (including mature trophozoites, schizonts, and gametocytes in *P. falciparum*, reviewed in [36]). Sequestered stages would not be present in samples of circulating parasite populations and hence would be omitted from the tally of infected RBCs (or stage percentage calculations).

Stage percentage: the percentage of sampled infected RBCs containing parasites in a particular developmental stage (e.g., % rings = $100 \times$ sampled parasites in the ring stage/sampled parasites in any stage of intraerythrocytic development).

Synchrony: the degree to which parasites within a population (*in vivo* or *in vitro*) complete intraerythrocytic development in unison. In a perfectly synchronous population, all parasites would burst out of RBCs at the same instant.

[20–22]. Such stage-specific drug action could select for parasite rhythms synchronized to drug-dosing schedules, akin to the ‘drug tolerance by synchronization’ predicted for viruses such as HIV [23]. Intriguingly, distinct drug resistance phenotypes have been generated by exposing asynchronous [24] versus synchronous [25] malaria cultures to **artemisinin**. Beyond drug resistance, altered developmental timing could help parasites tolerate changes in mosquito-biting rhythms following insecticide use (reviewed in [26]). Therefore, resolving the adaptive significance of synchrony – and the variation in parasite developmental schedules – is crucial to projecting parasite responses to control efforts.

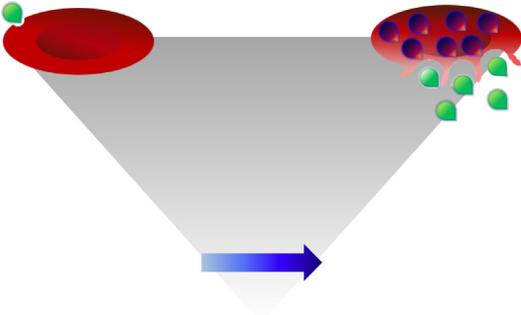
Trophozoite: the penultimate stage of intraerythrocytic development, following the ring stage and preceding the schizont stage, requiring approximately one quarter of the cycle length [19].

How Synchrony Fits into the Malaria Life Cycle

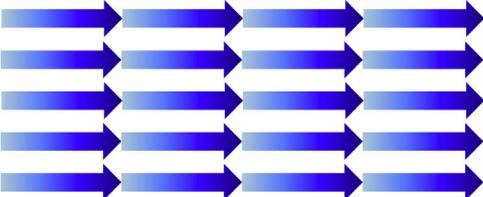
Malaria infections begin when a vertebrate host is bitten by an infectious Dipteran insect (mosquitoes for human and rodent malaria parasites). Upon inoculation into the bloodstream, parasites migrate to the liver, where they replicate before emerging to initiate blood-stage infection. This blood stage is the potentially symptomatic and transmissible phase of infection where the development of parasites within each cycle of asexual replication can be more or less synchronized. The timing of emergence from the liver could predispose parasite populations to synchrony or asynchrony: if parasites emerged rapidly from the liver to infect RBCs, the first cohort of intraerythrocytic parasites (that is, the first generation of infected RBCs) would exhibit a narrow range of developmental stages. In contrast, if emergence from the liver were prolonged, the first cohort would span a wide range of developmental stages. Only gradual release of parasites from the liver has been reported so far [27,28], in two rodent malaria species thought to be asynchronous [29,30].

Once parasites have invaded RBCs, they either multiply into numerous **merozoites** specialized for RBC invasion and further cycles of replication, or develop into **gametocytes** that can be transmitted to a vector ([31], reviewed in [32]). Merozoites are viable for only minutes once they burst out of RBCs [33], but **intraerythrocytic development** requires many hours – often a multiple of 24 that depends on the parasite species [11,34]. Parasites in RBCs initially resemble rings (hence **ring stage**), then develop into **trophozoites** as DNA synthesis begins, and finally **schizonts**, as replicated DNA is packaged into the merozoites that will burst out and continue the next round of RBC invasion (reviewed in [35]). Mature stages of some parasite species sequester in the host microvasculature (reviewed in [36]). Since those stages are more prone to being removed by the spleen [37], **sequestration** may represent a parasite adaptation to avoid clearance ([38], reviewed in [39]). In practice, sequestration means that a potentially large fraction of parasite biomass may not be apparent in a blood sample, and the parasites that are circulating will be biased towards nonsequestering ring stages [36].

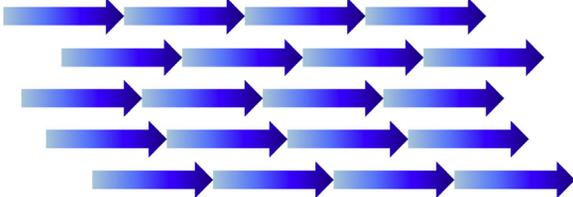
Developmental synchrony will therefore be influenced by two processes: (i) the time interval over which the first round of merozoites invade RBCs; and (ii) the variation in the time required for intraerythrocytic development (the **cycle length**). If there is no variation in cycle length, the level of synchrony is determined entirely by whether emergence from the liver occurs over a brief or extended period (Figure 1A,B). If cycle lengths vary across the population of infected RBCs, then the level of synchrony can change over time, as observed *in vitro* [17], illustrated in Figure 1C. Synchrony can be generated or maintained by constricting variability in cycle length (Figure 1D), as is done *in vitro* using heparin to inhibit invasion except during specific windows (e.g., [33]). Another possibility is that, within the host, parasites adjust their developmental timing to ensure synchronous emergence from RBCs [40], just as precociously developing periodical cicadas delay emergence so that slower members of their cohort can catch up (reviewed in [41]). Therefore, both host and parasite could modulate the realized variation in developmental timing and hence synchrony.



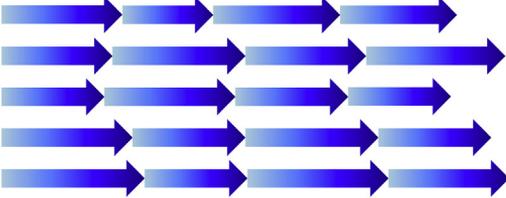
(A) Synchrony maintained



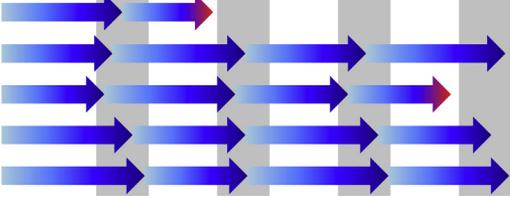
(B) Asynchrony maintained



(C) Decaying synchrony



(D) Enforced synchrony



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Stage Percentage–Based Methods Are Commonly Used and Biased

Among the methods used to assess synchrony (see Table I in Box 2), the most widely used, both *in vitro* [19,42,43] and *in vivo* [29,30,44,45], is the fraction of parasites in a particular developmental stage. Yet **stage percentages** can be biased by replication rates, a problem we illustrate with a heuristic model (see Figure 1 in Box 3). The key assumption in this heuristic model is that cycle length stays constant, so the level of synchrony is maintained through time and depends only on initial conditions (Figure 1A,B). Therefore, none of the scenarios we examine include alterations to the duration of intraerythrocytic development. Nonetheless, stage percentages fluctuate depending on population dynamics, independently of any difference in synchrony (Box 3). Specifically, expanding populations are enriched for early developmental stages, since each mature individual is – by definition – more than replacing itself. Contracting populations are biased towards later developmental stages. In either case, population dynamics perturb stage percentages away from the values expected to reflect the true level of synchrony.

How Large Is the Bias Due to Replication?

Perhaps reassuringly, even when parasite populations are expanding, the perfectly synchronous population undergoes more dramatic fluctuations than the asynchronous one (see Figure 1: C versus D in Box 3), raising the question of whether the bias in stage percentages due to replication is large enough to generate the spurious appearance of variation in synchrony, or obscure true differences in synchrony. Our heuristic model shows that the bias from realistic replication rates can be substantial. We find that the percent early rings can reach nearly 75% in a completely asynchronous population that is expanding eightfold, or can fall below 25% in a declining asynchronous population (Figure 2A). Realistic variation in replication rates generates the appearance of substantial variation in the level of synchrony where no variation exists. If we, instead, compare infections with polar opposite levels of synchrony but identical (eightfold) replication rates, it becomes nearly impossible to distinguish between a perfectly synchronized infection, perfectly measured, with a completely asynchronous one (Figure 2B). Realistically, most infections are likely to fall between the two extremes of perfect synchrony and complete asynchrony, and most treatments would fall short of inducing a switch to perfect synchrony. In combination with realistic experimental noise, parasite population dynamics make it difficult to distinguish even between polar opposite levels of synchrony. Therefore, the ability to distinguish any synchrony-related differences in stage percentages depends on the replication rate, which varies considerably across species and strains [4,13,19,46] and over the course of infection [19,46–53]. Cultured *P. falciparum* parasites retain enormous replicative capacity and must be diluted frequently to maintain viable cultures [17]. Different culturing protocols alter parasite replication rates, often by design (e.g., shaken cultures enhance multiplication rates 2.4-fold over static conditions [43]). The key point is that such large variation in replication rates could generate substantial differences in stage

Figure 1. How Synchrony Can Change (or Not) over the Course of Infection. The level of synchrony depends on the starting stages present in the parasite population and on the realized variability in the cycle length, where development within a red blood cell (RBC) is represented by a blue arrow. Each panel follows a population beginning with five parasites, assuming for the sake of visual clarity that those numbers do not increase through time. (A) Parasites that begin blood-stage infection at the same instant and exhibit no variation in the time required to mature inside RBCs will maintain complete synchrony through time. (B) When cycle length is invariant, asynchrony will be maintained when parasites initiate blood-stage infection at different points in time. (C) A population with a synchronous start (i.e., all parasites invade RBCs simultaneously) can lose synchrony through time if cycle lengths vary. (D) Synchrony can be maintained if the realized variation in cycle lengths is limited, for example, if parasites that burst outside a window of opportunity (gray rectangles) cannot invade RBCs (indicated by red-tipped arrows). The realized variation in developmental timing (here, the length of the arrows) has the potential to be influenced by both host and parasite factors.

Box 2. Methods for Detecting Synchrony

A variety of methods have been developed to identify synchronous infections (Table I), but many were never intended to make comparisons across different host-parasite combinations. For example, periodic fevers are a useful signal of synchronous dynamics in humans, but individuals vary considerably in the number of parasites required to trigger fevers (the 'pyrogenic threshold' [2]). Among humans who present without fever or other symptoms, synchrony has been identified from periodicity in parasite abundance [12]: since mature *P. falciparum* sequesters in the microvasculature, observed numbers of parasites can fluctuate dramatically over the 48 h replicative cycle in a synchronous infection, and individual parasite genotypes can be detected by PCR on alternate days. In asynchronous infections, parasite genotypes and numbers are more likely to be consistently detectable [12]. The abundance of particular genotypes may fluctuate substantially in ostensibly synchronous infections, while remaining constant in (presumably) poorly synchronized ones [16]. The level of synchrony has been quantified by fitting simple models to time series data (e.g., historical time series from neurosyphilis patients [4,13]). Periodicity in parasite abundance (or in the abundance of particular genotypes) is useful for detecting synchrony in *P. falciparum*. However, those same methods cannot detect synchrony in *P. vivax* – reported to be more synchronous than *P. falciparum* [2] – because mature parasites do not sequester in appreciable numbers [84]. These methods would likewise fail when host physiology is less permissive to sequestration (e.g., *rag1*^{-/-} mice [38]), or when parasites are artificially cultured and cannot sequester (nor cause fever, for that matter). Thus, periodic fluctuations in host temperature, the detectability of particular genotypes, or parasite numbers serve as useful signs of synchronous infections, but the absence of those signals need not indicate an absence of synchrony. Further, stage percentages represent the only method that can be used either *in vivo* or *in vitro*.

Table I. Existing Methods for Inferring Synchrony

| Metric | Biological explanation | Relevance | Refs |
|----------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------|------------------------|
| Periodic fever | Bursting of parasites from RBCs triggers fever, and duration of fever indicates duration of bursting (longer periods of continuous fever suggest poorer synchrony) | <i>in vivo</i> | [2,85] |
| Periodic fluctuations in abundance of parasite genotypes | Synchronously developing parasite genotypes periodically sequester where they cannot be detected | <i>in vivo</i> | [12,16,84,86] |
| Fit a sine wave to periodic fluctuations in parasite abundance | Amplitude of sine wave suggests degree of synchrony | <i>in vivo</i> | [13] |
| Fit a model to periodic fluctuations in parasite abundance | Variation in cycle length fitted as a parameter | <i>in vivo</i> | [4] |
| Stage percentages | A larger percentage of parasites in a particular stage is thought to indicate greater synchrony | <i>in vivo</i> and <i>in vitro</i> | [19,29,30,43–45,56–59] |

Box 3. A Heuristic Model for Tracking Parasite Maturation

We envision a parasite species where development requires 24 h (e.g., *P. chabaudi*) and each stage takes 6 h to complete, but the times are arbitrary: parasite population dynamics will vary depending on when the population is sampled, and that will influence stage percentages. Following invasion, parasites progress through the early ring stage (*E*), the late ring stage (*L*), the trophozoite stage (*T*), and finally the schizont stage (*S*):

$$E_t = BS_{t-1} \quad \text{[I]}$$

$$L_t = E_{t-1} \quad \text{[II]}$$

$$T_t = L_{t-1} \quad \text{[III]}$$

$$S_t = T_{t-1}, \quad \text{[IV]}$$

where the subscript *t* tracks time steps (here 6 h). We assume that the number of merozoites that emerge from each schizont and successfully invade new RBCs (*B*) is constant over each developmental cycle. For conceptual clarity, we assume that development consists of four stages of equal duration and track a small parasite population initially consisting of four individuals. However, the model can be represented as a matrix to project population dynamics from an initial stage distribution for any arbitrary number of developmental stages ([87], reviewed in [88]). We modulate *B* to examine the impact of replication rates (Figures 2 and 3D,E), or group early and late rings together to mimic the inability to distinguish different stages (Figure 3B). Finally, to simulate the impact of sequestered (and therefore unsampled) mature stages, we omit schizonts from the calculation of percent early rings (i.e., we set percent early rings = $E(t)/[E(t) + L(t) + T(t)]$, Figure 3C).

We first compare two extremes of synchrony: (i) a perfectly synchronized population, where all members of the parasite population begin in a single developmental stage; (ii) a completely asynchronous population, where each parasite is in a different developmental stage initially. Assuming parasite numbers remain constant ($B = 1$), the percent early rings fluctuates between 0 and 100 in the synchronous population but remains unchanging at 25% in the asynchronous population (Figure 1A,B). The clear differences suggest that – at least for extreme differences in synchrony – stage percentages could serve as useful metrics (for example, a high percentage of rings at a single time point [44]; or a high percentage of rings at a certain time of the cycle [43]; or using the standard deviation in stage percentages [29,30]). However, when parasite populations expand twofold ($B = 2$), both synchronous and asynchronous populations exhibit periodic fluctuations in the percentage early rings (Figure 1C,D). The percentage of any stage would be expected to fluctuate periodically when parasite numbers are changing, even in the absence of synchrony.

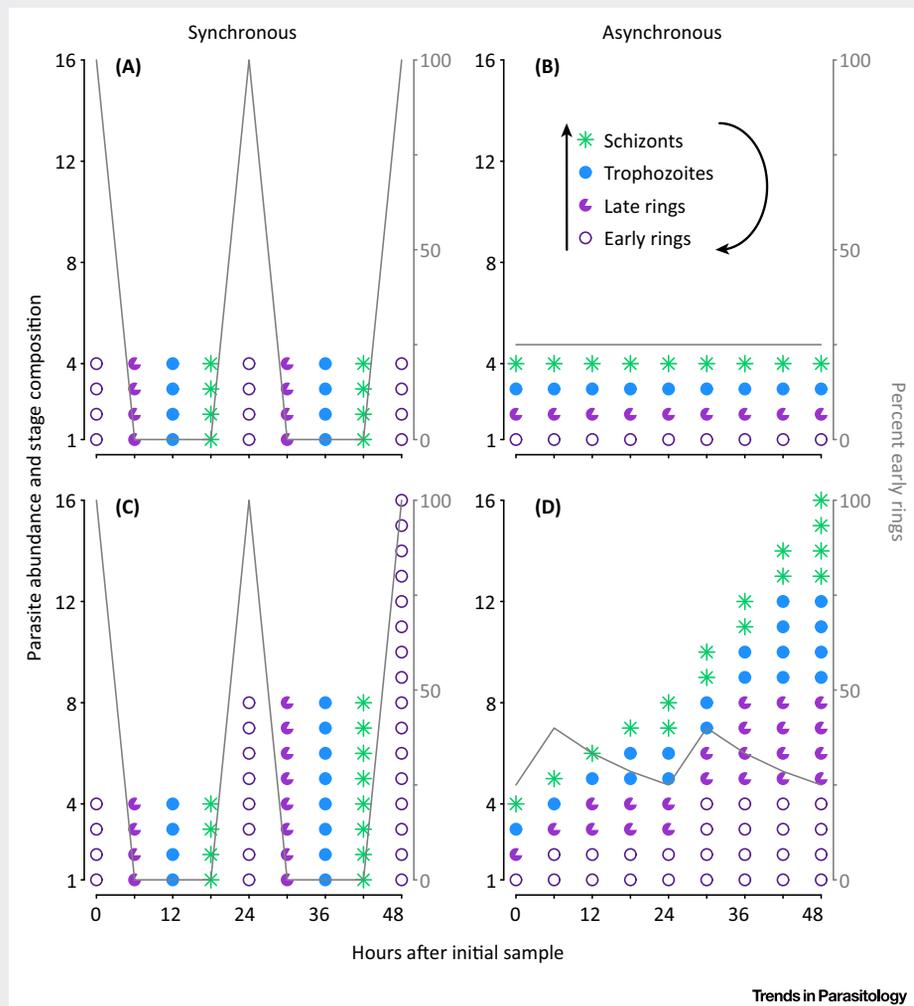


Figure 1. Population Dynamics Bias Stage Percentages. Each population begins with four parasites either all in a single stage ('synchronous', A, C) or each in a different stage ('asynchronous', B, D). Early ring percentages match expectations for perfect synchrony (A) or asynchrony (B) when parasite numbers are constant. When the parasite population is expanding twofold, the percent early rings fluctuates whether the population is synchronous (C, percent early rings identical to the population shown in (A) or asynchronous (D).

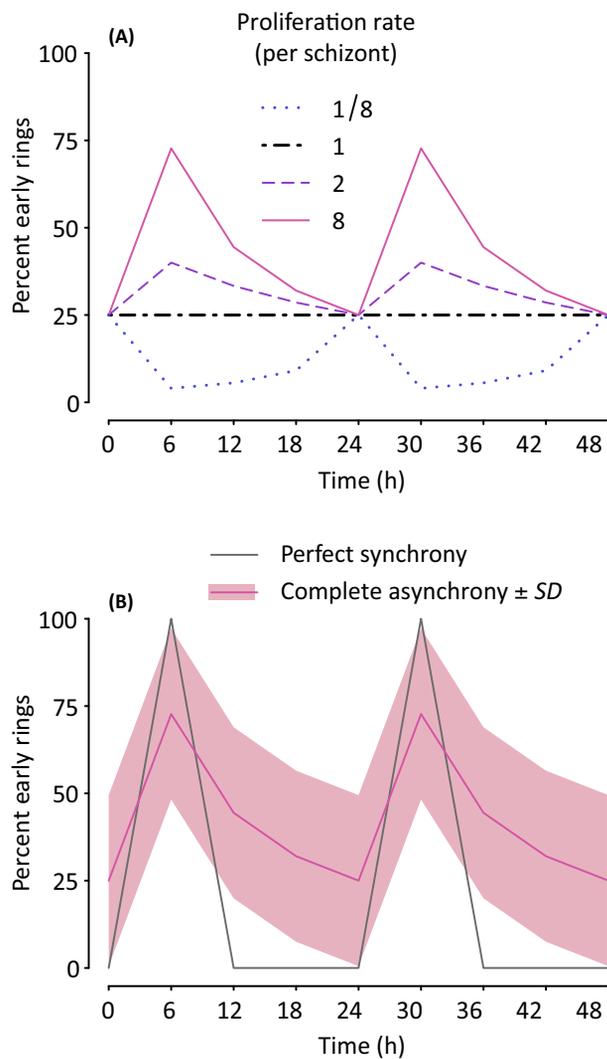


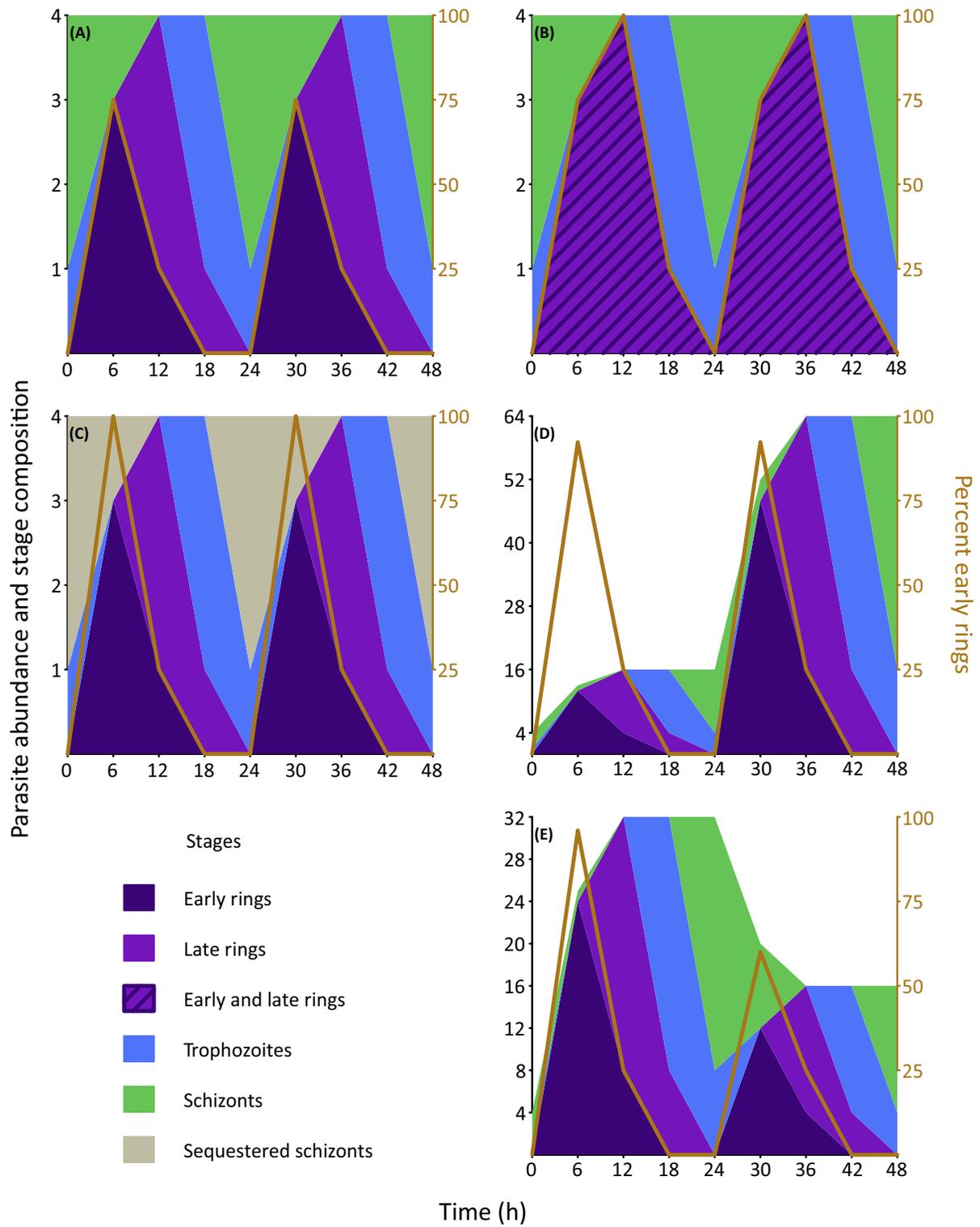
Figure 2. Population Dynamics Obscure Synchrony. (A) Asynchronous populations can exhibit large fluctuations in stage percentages, depending on replication rates, giving the appearance of varying degrees of synchrony. If populations expand eightfold ($B = 8$), consistent with reports from *Plasmodium falciparum* *in vivo* [4,13] and *in vitro* [19], early ring percentages can reach nearly 75%, even in an asynchronous population (pink solid line). The minimum percent early rings can dip below 25% in a contracting population (blue dotted line). (B) Holding replication constant ($B = 8$), polar opposite levels of synchrony can appear nearly identical given realistic standard deviation in stage percentages. The clearest picture of variability inherent in stage percentages comes from *rag1*^{-/-} mice, which are impermeable to parasite sequestration; asynchronous infections of those mice exhibit standard errors of nearly 10% in stage percentages across six replicate mice (Figure 1C in [38]), equivalent to a standard deviation of almost 25% as plotted here. We plot the perfectly synchronous infection as if it were perfectly measured, though in reality these estimates would also be subject to error.

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percentages, even with no change to the duration of development or differences in synchrony. While noise in stage percentages may be lower in tightly controlled *in vitro* conditions [43], a method that can only distinguish between levels of synchrony in *P. falciparum* *in vitro* – and only under the special condition that different treatments have identical population dynamics – will not be able to make the broader comparisons necessary to identify proximate and evolutionary drivers of synchrony.

Differences in Replication Rates Are Not the Only Source of Bias

Real infections are unlikely to be perfectly synchronous, and even malaria species considered synchronous (e.g., *P. chabaudi*) have some variation in the timing of bursting [54]. We next consider a population of parasites that is highly (but not completely) synchronous, and examine the impact of two other potential sources of bias: inability to distinguish or to sample different developmental stages (Figure 3). Fluctuations in ring percentages can resolve the correct level



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of synchrony when parasite numbers are constant, when all four stages are distinguishable, and none sequester where they cannot be sampled (Figure 3A). Specifically, the percentage of early rings fluctuates between zero and 75%, as would be expected in a population that began with 75% of parasites in a single developmental stage (compare with a completely synchronous or asynchronous population, see Figure 1A,B in Box 3). However, it is overly simplistic to assume that distinguishable developmental stages will always be of equal duration, or that the identical developmental stages will be distinguishable across diverse host–parasite combinations. When early and late rings cannot be distinguished (Figure 3B), the fluctuations in ring percentages appear nearly identical to the perfectly synchronized case. The more extreme oscillations result from the fact that the combined early and late ring stage is twice the duration of the other stages. Thus, the expected stage percentages depend on the duration of each stage, data that are challenging to obtain. Further, the duration of a particular stage may vary with the strain in question (e.g., ring-stage duration across different cultured *P. falciparum* strains [19]). The same parasite population could also appear more synchronous if mature stages sequester (Figure 3C), or if a treatment also changes the duration of development (and hence the timing of sequestration). Though we assume in this example that only schizonts (i.e., parasites in the last quarter of development) sequester, in reality parasites in the latter half of development are prone to sequestration ([4], reviewed in [36]), potentially generating even more extreme bias towards early stages. Parasite replication can also lead to more extreme fluctuations in early ring percentages in highly synchronized populations (Figure 3D), just as it does for an asynchronous population (see Figure 1D in Box 3). We also examine the case when replication rates decrease through time, as would be expected in a real infection, and show that the apparent level of synchrony – as gauged by the percent early rings – likewise declines (Figure 3E). Therefore, stage percentages data are subject to several sources of bias, including the ability to morphologically distinguish different stages, uncertainty in stage durations, sequestration of mature stages, and differences in parasite replication rates.

Do These Biases Matter?

Stage percentages produce biased estimates of synchrony, but we argue that the significance of that bias depends on the goal of the study. Frequently researchers need parasite cultures enriched with a particular developmental stage – for example to quantify stage-specific activity of drugs [20] or immune effectors [55] – and it makes no practical difference whether that stage enrichment was obtained by changing replication rates or altering the timing of development, or both. However, if the goal is to identify treatments that reduce variation in cycle length and thereby enhance synchrony (Figure 1D), using stage percentages is fraught with difficulty even in the comparatively simple case of artificial culture. Notably, improved methods of enhancing developmental synchrony *in vitro* continue to be sought [56,57] because asynchrony introduces an additional source of noise. For example, detecting stage-specific action *in vitro* – especially for chemicals with short half-lives, such as artemisinin – requires extremely high levels of synchrony [58]. Improvements to *in vitro* protocols could proceed faster with robust methods for quantifying synchrony.

The current best practice is to rule out treatment-induced changes to population dynamics before making use of stage percentage data, as has been done while examining the timing of

Figure 3. Stage Percentages Are Biased by Multiple Processes. With the same heuristic model, the population begins as one trophozoite and three schizont-stage parasites, a well synchronized, but not perfectly synchronized, population (cf Figure 1 in Box 3). When parasite numbers are constant, all stages are morphologically distinguishable and none sequester, early rings fluctuate between zero and 75% as expected (A). Fluctuations in ring percentages are more extreme – giving the appearance of greater synchrony – when early and late rings cannot be distinguished (B), when schizonts sequester and cannot be detected (C), and when the parasite population is expanding (here twofold, D). Finally, if per schizont replication falls from eight to one-half in the first versus second cycle (E), the peak percent of early rings decreases, giving the illusion of declining synchrony.

host feeding in experimental rodent infections [6]. That approach minimizes the chances of finding spurious differences in synchrony (Figure 2A), but parasite population dynamics can still influence the ability to distinguish between truly different levels of synchrony (Figure 2B). Further, that approach fails when the treatment alters parasite replication in addition to stage percentages (e.g., melatonin treatment of mice [59], shaking of *in vitro* cultures [43]). More importantly, extrapolating from *in vitro* stage percentages to real infections, where several additional sources of bias come into play (Figure 3), is highly questionable. If the ultimate aim is to disentangle the drivers of synchrony within hosts, that requires making comparisons across infections and over the course of infection, and stage percentage data alone are too profoundly biased to be useful.

What Data Are Needed? Insights from Other Systems

Understanding the evolutionary drivers and public health relevance of synchrony requires knowledge of within-host ecology which is poorly captured by stage-percentage data. Rather than percentages, parasite population dynamics depend on the number of parasites in particular developmental stages (e.g., resource competition, immune responses; Box 1). Obtaining accurate counts of each developmental stage is challenging, especially if mature stages sequester, but those challenges are not unique to parasites. Like malaria parasites, insects interact with their environment in a stage-specific manner, and the inability to accurately sample all developmental stages is a common obstacle. Nonetheless, key details of ecology, including resource competition between different stages and vulnerability to natural enemies, have been inferred from time series of adult abundance (e.g., [60]), as have the drivers of synchronized outbreaks [61]. In both cases, models track unobservable developmental stages, eliminating the need to quantify abundance of all stages. Thus, tracking infected RBC abundance, rather than stage percentages, is more likely to be useful for examining developmental synchrony in malaria parasites.

Obtaining accurate infected RBC counts presents a few difficulties. Modern PCR methods quantify genome copy number, which only reflects infected RBC abundance when the parasite population is synchronized and has not begun the process of DNA replication [62]. In contrast, microscopic examination of thin blood smears could give an accurate count of infected RBCs, but reflects only those that are circulating and not sequestered. When sequestration patterns are known in advance, simple within-host models have been used to reconstruct total infected RBC abundance and quantify synchrony [4]. When sequestration patterns are not known, or likely to vary within a data set, models can account for dynamics of unobservable developmental stages (e.g., insect eggs [63,64]). New techniques use biomarkers [65,66] or bioluminescence [67,68] to quantify total parasite biomass, but the expression of these markers changes over the course of parasite development, making it nontrivial to infer infected RBC abundance. For quantifying synchrony, the best approach is likely tracking circulating infected RBC abundance from microscopy and using models to account for sequestration.

In making comparisons across diverse parasites, an ideal method would be robust to differences in cycle length. Cycle lengths are not always known, and vary across species [34], strains [69], and potentially even over the course of infection [70]. Notably, the methods used to quantify cycle length also commonly rely on stage percentage data and could therefore be biased by the same processes we have identified here (Figure 2). Yet the problem of identifying cycle length – and changes in cycle length – is not fundamentally different from, for example, quantifying the lag between recurrent outbreaks from incidence data and determining whether the lag is changing through time. That problem has been addressed through wavelet decomposition (e.g., from time series of pertussis cases [71], and

adult moths [61]). Applying those techniques to parasite population dynamics within the host could prove illuminating.

The length of time series data influences the ability to detect differences in synchrony. Malaria studies commonly rely on short time series consisting of 1.5–4 developmental cycles intensively sampled [19,29,30,45,54], or occasionally even a single time point [44], but longer time series are more informative. Two decades of data were used to quantify geographic differences in the periodicity of synchronized reproduction in rowan trees (2- versus 3-year cycles [72]). Typically, time series include short developmental or reproductive cycles layered onto long-term population dynamics; in the context of a synchronized malaria infection (e.g., Figure 1C in Box 3), there would be periodic spikes in parasite number resulting from synchronized bursting layered on top of a general trend of changing abundance that occurs whether the population is synchronized or not. This complexity is apparent from our heuristic example, where the perfectly synchronous and asynchronous populations attain the same numbers at 0, 24, and 48 h despite large differences in the short-term dynamics (see Figure 1C,D in Box 3). Long-term trends must be filtered out to distinguish the signal of developmental synchrony, but linear trends are all that can be discerned and removed when time series are short compared to the developmental cycle (for example, 26 years of data compared with 13- or 17-year cycles of synchronous cicada emergence [73]). Past investigations into human malaria dynamics have focused on the acute portion of infection where parasites replicate in a log-linear manner that is straightforward to filter out from the periodic signal of synchronized parasite development [13], and this approach could prove useful for time series that are short by necessity (e.g., human vaccine trials [74]).

An alternate approach is to leverage longer time series to filter out more complicated long-term dynamics and isolate signals of synchrony (and of changing synchrony) that would not be apparent otherwise. For instance, tea tortrix moths undergo synchronized outbreaks – at times, multiple per season – in addition to seasonal expansion and decline [61]. Nelson *et al.* [61] used 51 years of data – encompassing about 250 cycles – to filter out seasonal fluctuations and identify temperature as a driver of these synchronized intra-annual outbreaks. Time series encompassing hundreds of developmental cycles cannot be obtained from modern human infections since even subclinical infections carry health risks if untreated [75], but historical time series exist [15] and portions of those time series have already been used to assess synchrony [13]. Comparing those historical time series with malaria infections in mice and *in vitro* – where time series of tens of cycles are obtainable – would improve understanding of how synchrony changes over the course of infection and whether that varies with parasite species or within-host conditions.

Towards Better Methods

Even in systems where abundant time series data are available, the ideal methods for quantifying synchrony are still debated. Three approaches show promise for comparing synchrony across insect species [64]. First, synchrony can be estimated by fitting mechanistic models that explicitly account for developmental time delays. Simple models have been fitted to malaria infection time series [4,13], a useful yet underutilized approach. More detailed models exist for malaria infections [3,76] that track gametocyte dynamics; fitted to data, these models could estimate synchrony and project transmission consequences. However, existing models assume that cycle length is fixed across a parasite population (Figure 1A,B); future research would benefit from models that allow changes in synchrony (Figure 1C,D). Second, circular variance could quantify synchrony as the variation in a developmental index based on stage abundance data [64], a method that could cope with

morphologically indistinguishable stages or stages with unequal lengths (Figure 3B). Unfortunately, it requires abundance for each stage and does not explicitly account for population expansion or contraction that can cause stage distributions to deviate from expectation (Figure 2). Third, wavelet analysis distinguishes cycles of different length from time series data (e.g., an annual cycle, a generation cycle, details in [61,64]). Though not yet applied to malaria data, this method could detect the signal of changing synchrony through time.

Beyond their role as a method for quantifying synchrony [4,13], mechanistic models of infection dynamics are needed to refine empirical approaches. Models could be used to simulate infection data – where the true pattern of synchronization is known – and test the efficacy of candidate approaches for quantifying synchrony (e.g., wavelet analysis). Further, models can identify optimal protocols for sampling infections. Long, high resolution time series provide more information, but there are practical constraints on sampling timing and frequency. Models are needed to simulate infections with realistic levels of sampling error (for example, error in RBC counts characterized from rodent malaria infections [77,78]). Incorporating plausible sampling error, *in silico* ‘experiments’ could identify the sampling schedule needed to detect differences in synchrony – and changing synchrony – from noisy data.

Concluding Remarks

Developmental synchrony in malaria parasites appears to vary across host and parasite combinations, motivating research to uncover the proximate and evolutionary causes of such diversity (e.g., [3,5–11,43,44,79–81]). The level of synchrony is not just a scientific curiosity but is also expected to influence how patients respond to treatment with antimalarial drugs [4], and, potentially, how parasites will evolve in response to drug treatment [3] since developmental stages show differential susceptibility to drugs (e.g., [20,22]). Yet the diverse methods used to assess synchrony have received far less attention, and those methods have important limitations. Many methods rely on quirks of biology specific to particular host–parasite combinations (see Table 1 in Box 2) and cannot be used to make comparisons. Stage percentage data are obtainable from infections *in vitro* and *in vivo* and would therefore appear ideal for making the kind of comparisons that would elucidate the drivers of synchrony. However, stage percentages vary with a large number of processes unrelated to the timing of development (Figures 2 and 3), and the bias introduced by those sources is rarely accounted for when quantifying the level of synchrony. Given the limitations of existing methods, it remains an open question to what extent parasites (or hosts) actually vary in their ability to maintain synchronous infection dynamics (see Outstanding Questions).

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Outstanding Questions

How does synchrony vary through time within infections?

- Do parasites become ‘regimented’ into synchronous development by immunity during acute infection?
- Does synchrony decline over time in chronic infections?
- Using models to simulate changes in synchrony, what length and resolution of time series data allow those changes to be detected?

How does synchrony vary across parasite strains?

- Do cultured *P. falciparum* strains exhibit heritable variation in their propensity to maintain synchronous development in identical *in vitro* conditions?
- Can synchrony be selected for (or against, for example, by different drug treatment regimens)?
- Do poorly synchronized strains replicate faster?

How does synchrony vary across hosts?

- Is synchrony more pronounced in naive or previously exposed hosts?
- Do some hosts maintain synchrony better than others following infection by the same parasite strain?
- How do host genetics interact with environment (for example, circadian rhythms in feeding) to modulate synchrony?

How does synchrony vary across host–parasite genotype combinations?

- To what extent is synchrony host-driven versus parasite-driven?
- If asynchrony hastens replication (see above: How does synchrony vary across parasite strains?), is it associated with worse host health outcomes?
- When (if ever) does synchrony enhance transmission success?

Does synchrony vary across parasite species?

- Does variation in synchrony across species dwarf the within-species variation?
- Does the variation in synchrony across different strains overwhelm temporal variation over the course of infection?
- How do different parasite species maintain such distinct schedules in spite of infecting a common host?

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