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## SUMMARY

Biological rhythms appear to be an elegant solution to the challenge of coordinating activities with the consequences of the Earth's daily and seasonal rotation. The genes and molecular mechanisms underpinning circadian clocks in multicellular organisms are well understood. In contrast, the regulatory mechanisms and fitness consequences of biological rhythms exhibited by parasites remain mysterious. Here, we explore how periodicity in parasite traits is generated and why daily rhythms matter for parasite fitness. We focus on malaria (*Plasmodium*) parasites which exhibit developmental rhythms during replication in the mammalian host's blood and in transmission to vectors. Rhythmic in-host parasite replication is responsible for eliciting inflammatory responses, the severity of disease symptoms, fuels transmission, and can confer tolerance to anti-parasite drugs. Thus, understanding both how and why the timing and synchrony of parasites are connected to the daily rhythms of hosts and vectors may make treatment more effective and less toxic to hosts.

## KEYWORDS

Circadian clock, circadian rhythm, *Plasmodium*, intra-erythrocytic development cycle, synchronicity, periodicity, entrainment, fitness, host-parasite interactions, nutrient sensing, metabolism, inflammatory response.

## Periodicity in malaria parasites

Malaria infections are frequently lethal, especially in children under 5 years of age, and 40% of the world's population live in endemic areas (World Malaria Report, 2019). Fever rhythms during malaria infection were first documented during the Hippocratic era, and later, the interval (periodicity) between fever bouts were used to diagnose the species of *Plasmodium* a patient was infected with. Fever is a direct consequence of the inflammatory response that is elicited when a cohort of asexually replicating stages synchronously burst out of the host's red blood cells (schizogony) to release their progeny (merozoites). Following release, merozoites invade more red blood cells (RBCs) to initiate a new cycle of asexual replication termed the "intra-erythrocytic development cycle" (IDC; Fig 1a and Fig 1b) (Gerald et al., 2011). Within every cycle, a small proportion of parasites commit to differentiating into sexual stages (termed "gametocytes") which are responsible for infecting insect vectors. Upon being taken up in a mosquito vector's blood meal, gametocytes rapidly differentiate into gametes and then mate. The offspring undergo extensive replication before eventually making their way to the salivary glands to be transmitted to new hosts.

The IDC's of most species of *Plasmodium* last for multiples of 24 hours (Mideo et al., 2013), suggesting a circadian basis. A flurry of interest several decades ago (stimulated by Hawking et al., 1968; 1970) proposed explanations for the rhythmicity observed in development during the IDC, but these hypotheses have proved hard to reconcile with recent observations. A better understanding of how the IDC schedule is controlled *in vivo* (Fig 1b) is necessary for several reasons. First, asexual replication underpins the severe symptoms of malaria infection (Gazzinelli et al., 2014) and fuels the production of gametocytes (Carter et al., 2013). Second, tolerance to antimalarial medications involves a period of dormancy during the IDC (Teuscher et al., 2010), suggesting plasticity in the IDC can be employed as a survival strategy. Third, reports of malaria-vectoring mosquitoes evading bed nets by altering the time of day they forage for blood suggests the temporal selective landscape of malaria parasites is changing (Thomsen et al., 2017).

Having an IDC that is coordinated to the host's circadian rhythm matters for parasite fitness (O'Donnell et al., 2011; 2013). However, why the parasites benefit from their IDC schedule, and how the IDC schedule is controlled, remain mysterious. Here, we outline how the integration of parasitology with chronobiology, evolutionary ecology and immunology, is uncovering how the IDC is scheduled and what its fitness consequences are for malaria parasites. Recent work has begun to understand how the daily rhythms exhibited by hosts and vectors impose challenges that parasites must cope with and, conversely, offer opportunities that parasites can exploit. We focus on *Plasmodium spp. (*malaria parasites), because their rhythms are the best understood, and draw inferences from other parasites where relevant. Recognizing that daily rhythms underpin infection processes could reveal times of day that parasites are particularly vulnerable to drug treatment, when drugs are least toxic, and how the host's rhythms might be harnessed to improve defense and recovery. We introduce the relevant concepts from chronobiology and evolutionary ecology, evaluate whether malaria parasites can keep time, then consider how the daily rhythms of hosts and vectors generate a highly dynamic and complex environment for parasites to navigate, before highlighting the major areas for future work.

## Chronobiology concepts

Like almost all organisms, parasites experience a rhythmic world (Reece et al., 2017; Rijo-Ferreira et al., 2017a, Westwood et al., 2019). Whilst inside vertebrate hosts, parasites are somewhat sheltered from rhythms in the "abiotic" external environment thanks to the homeostasis of the host. However, within the host, they are confronted with a myriad of daily "biotic" rhythms in behaviors, physiologies, and cellular processes driven by the circadian clocks of their host (Fig 1, Pittendrigh, 1960; Reece et al., 2017). For example, rhythms in immune defenses may make it dangerous for parasites to perform certain activities at a

particular time of day, and rhythms in host foraging may result in resources only being abundant at certain times of day. Whilst it is not clear why malaria parasites exhibit a rhythmic IDC (a *Plasmodium* clock has yet to be discovered), their hosts and vectors benefit from using circadian clocks to govern many behaviors and physiologies. Organisms are thought to garner fitness benefits from coordinating with rhythms in the external environment ("extrinsic adaptive value") and from temporally compartmentalizing incompatible physiological or cellular processes ("intrinsic adaptive value") (Sharma, 2003). For example, experiments using cyanobacteria and *Arabidopsis* reveal that rhythms matching the duration of the light-dark cycle provide an advantage over competitors whose rhythms have a different duration (Ouyang et al., 1998, Dodd et al., 2005).

Most of the daily rhythms exhibited by mammalian hosts and insect vectors are driven by circadian clocks. Across disparate species, the canonical clock mechanism shares a similar design (Fig 2a), but the specific genes and proteins involved are distinct (Dunlap, 1999). The common feature across different taxa is the presence of a self-sustaining transcription-translation feedback loop (TTFL), operative within individual cells. For instance, in mammals, heterodimers of basic helix-loop-helix transcription factors produce transcriptional activation of target genes which include the *Period* (*Per1-Per3*) and *Cryptochrome* (*Cry1- Cry2*) genes. Protein products to the *Per* and *Cry* genes feed-back to repress their own expression, providing a molecular feedback loop with a cycle length of approximately 24 hours (Reppert and Weaver, 2002, Fig 2a, 2b). An interlocking feedback loop of additional transcription factors stabilizes and enhances the core clock loop. Chromatin remodeling enzymes, other transcription factors, and proteins, affect the activity and stability of these core clock proteins (including casein kinases, protein phosphatases, and several F-box proteins), influencing cycle length and gene expression rhythms (Takahashi et al., 2015). Additional levels of regulation abound, with post-transcriptional and post-translational regulatory mechanisms now well-established (Koike et al., 2012; Kojima and Green, 2015). Studies in mouse tissues reveal that as many as 40% of genes are rhythmically expressed in at least one tissue (Zhang et al., 2014). The expression of circadian clock-regulated genes exhibit periods (durations) of ~24h, and their phase/ amplitude serve as useful parameters to compare rhythms across experimental groups (Fig 2b). Circadian clocks are also temperature compensated, enabling them to tick at the correct pace across a biologically relevant temperature gradient (Fig 2c). Clock-regulated genes often include key, rate-limiting steps in biological and metabolic processes (Panda et al., 2002) and include many targets for top-selling pharmaceutics (Zhang et al., 2014; Ruben et al., 2018). The mammalian circadian clock thus casts a pervasive influence on the function of numerous cell types and tissues, including important effects on metabolism (Bass, 2012; Sancar and Brunner, 2014; Rijo-Ferreira & Takahashi, 2019).

Mechanisms for circadian rhythmicity also exist which are *not* dependent upon the known TTFL genes and mechanisms. Biochemical (redox) rhythms in human erythrocytes occur *in vitro* despite the absence of transcription (O'Neill and Reddy, 2011). These redox rhythms are thought to be mediated by rhythmic ion transport (Feeney et al., 2012; Henslee et al., 2017) and are evolutionarily ancient (Edgar et al., 2012). Precisely how different types of clock interact, and how clocks situated in different organs throughout an organism are coordinated, are unclear. In mammals, TTFL clocks situated in the suprachiasmatic nucleus of the brain (the SCN, also known as the central or "master" clock) relay light-dark cycle information to peripheral clocks, and peripheral clocks also schedule ("entrain") to other rhythmic events such as feeding (Fig 2d). Thus, the daily rhythms of hosts (and vectors) generate a highly dynamic and complex environment for parasites to navigate.

**Interrogating the IDC schedule**

In the context of explaining how host-parasite interactions shape the IDC, most work focuses on identifying the genes or molecular pathways that determine IDC progression. Here, we advocate including an evolutionary ecology framework. This framework considers how

interactions (both within/between species and with aspects of the environment) shape the traits exhibited by organisms through adaptation and selection. Coevolution recognizes that the consequences of evolutionary change to a parasite trait may impose selection on host/vector traits, and vice-versa. In the context of this paper, evolutionary ecology poses the questions “to what extent, and why, is natural selection acting on parasites, hosts and vectors responsible for shaping the IDC schedule” (Fig 3)? Answering these questions involves deconstructing the IDC into quantitative traits that natural selection could act on and asking how parasite ecology affects the costs and benefits garnered from different values that IDC traits could plausibly take. Put another way, could the IDC exhibit different timing and degrees of synchrony (trait values)? If so, is the observed IDC schedule the one that returns the highest possible fitness in terms of within-host survival and between-host transmission? If not, why don’t parasites exhibit the “best” IDC schedule? Furthermore, if there is variation between genotypes/species in the IDC schedule, does this mean that natural selection has failed to hone all genotypes/species to the “best” IDC schedule, or do the differences between genotypes/species call for different IDC schedules? In terms of IDC traits that can be readily quantified, both the degree of synchrony within each IDC cohort and the times of day at which developmental transitions between IDC stages occur require explanations (Mideo et al., 2013). When considering correlated traits, it is important to ascertain whether both traits confer benefits and if so, whether they are independently favored by natural selection (Fig 3c). Alternatively, perhaps only one trait is selected for and the other occurs as a by-product (Fig 3a, 3b), or neither of the traits are beneficial to parasites (Fig 3d). It is also important to recognize that the selective pressures driving the emergence of a trait may not be the same selective forces responsible for its maintenance.

Another consideration is which party is in control of the trait(s) in question: i.e. to what extent do genes belonging to the host and/or parasite control the IDC schedule? Further complexity arises because the balance of host and parasite influences may alter during infections due to the dynamic nature of immune responses, disease symptoms, and parasite densities (Prior et al., 2019; Rijo-Ferreira et al., 2018). If the IDC is coordinated by a mechanism(s) encoded by parasite genes, then parasites – for better or for worse for their fitness - are actively in control of their IDC schedule. Alternatively, parasites may have an intrinsically arrhythmic IDC and allow host circadian rhythms to impose a schedule that coincidently benefits parasites (Fig 3d). The distinction between host and parasite control is subtle but disentangling to what extent each party is in control of the IDC schedule, and the costs and benefits they receive, is useful for several reasons. First, it helps narrow down the search for genes and molecular mechanisms that underpin traits expressed during the IDC to the correct party. Second, changes to parasite ecology (*e.g.*, as a consequence of a shift in mosquito biting time) may alter the balance of costs and benefits of a particular IDC schedule. Whether parasites can counter-evolve depends on how much of their genes influence the IDC schedule. Third, quantifying how much variation in parasite alleles affects variation in IDC trait values allows predictions to be made for the rate and direction of parasite evolution. The data discussed in the following sections suggests the IDC schedule is a product, at least to some extent, of parasites keeping time, but is also strongly influenced by host circadian rhythms.

IDC rhythms can be interrogated using species such as *Plasmodium chabaudi,* whose asexual stages develop during the IDC in synchrony and transition from one stage to the next at particular times of day (Fig 1a and 1b). Because *P. chabaudi* is an *in vivo* model, its IDC can be studied in a more ecologically realistic setting than *in vitro* models. *P. chabaudi’s* IDC lasts approximately 24 hours and different IDC stages can be distinguished on blood smears by their morphology. However, detecting later IDC stages is challenging because, like the human malaria parasite, *Plasmodium falciparum,* late trophozoites and schizonts sequester in the host tissues via cytoadherence to endothelial cells until schizogony is completed (Mackintosh et al., 2004; Miller et al., 2002). The following sections illustrate that coupling the ecological complexity of an *in vivo* model with the reductionism possible with an

*in vitro* model, such as *P. falciparum,* offers a powerful way to integrate both the proximate ("how" or mechanistic) with the ultimate ("why" or evolutionary) explanations for the IDC schedule.

**Can parasites keep time?**

While there is clear evidence for timing mechanisms in some parasite taxa, evidence that malaria parasites can organize their IDC schedule is suggestive and indirect. The infectious agent of sleeping sickness, the *Trypanosoma brucei* parasite, has a circadian clock that controls the timing of expression of over 1,000 genes, mostly associated with its metabolism (Rijo-Ferreira et al., 2017b). The timing of these rhythms is entrained *in vitro* by temperature, suggesting that *T. brucei* actively schedules its daily activities in relation to the active (warm) and rest (cool) phases of the host's circadian rhythm. Because animals forage and undertake most metabolism in their active phase, aligning its rhythms with temperature may allow *T. brucei* to coordinate with host feeding events. The fungal pathogen *Botrytis cinerea* has a circadian clock which regulates how virulent it is to its *Arabidopsis thalania* hosts, allowing it to overwhelm host defenses that are upregulated at dusk (Hevia et al., 2015; Larrondo and Canessa 2018). Thanks to work on the model fungus *Neurospora crassa*, the components and operation of the *Botrytis* clock are known. However, neither of the parasites *Trypanosoma* or *Plasmodium,* possess any genes homologous to the "clock genes" described in *Neurospora*, cyanobacteria, mammals, or fruit flies. Thus, if malaria parasites have a circadian oscillator, one option would be a classical TTFL operated by novel clock genes.

Conventional methods for searching for an oscillator are difficult to apply to *P. chabaudi* because genome-wide screening approaches require robust and self-sustaining oscillations *in vitro,* while approaches based on rhythmic gene expression of parasites *in vivo* are inevitably confounded by synchronous development throughout the IDC of ~24 hours. Using *P. falciparum* would overcome some of these obstacles because its IDC duration is 48 hours and it can be cultivated *in vitro* (Subudhi et al., *biorXiv*). Thus, experiments in which constant ("free-running") conditions are generated by either not replenishing or continuously replenishing media, could use *P. falciparum* to test for 24-hour rhythms in gene expression and protein production, as well as temperature compensation. Such experiments are necessary because observations from *P. falciparum* and *P. chabaudi* are not obviously consistent with a circadian clock. For example, the IDC rhythms of *P. falciparum* break down readily in culture (Schuster, 2002); the duration and synchrony of *P. chabaudi's* IDC alters when hosts are sick during the peak of infection (Prior et al., in prep); and completion of the IDC across *Plasmodium spp.* can be slowed by a reduction in temperature (Rojas and Wasserman, 1993). However, using observations based on IDC development to reject the presence of a circadian clock is premature. If these conditions de-couple the ability of a clock's readouts to schedule the IDC, then a disrupted IDC does not indicate the absence of a clock. For instance, perhaps a clock keeps on ticking with a 24-hour duration, despite the IDC being slowed by cooling.

Instead of a sophisticated circadian oscillator, such as a TTFL, parasites may keep time using a rudimentary clock. For example, an "hourglass timer" (whereby the hourglass is "turned" when a signal is received) would allow parasites to set the IDC schedule on detection of a time-of-day signal in the environment, but would not generate self-sustaining oscillations (Pittayakanchit et al., 2018). An even simpler strategy would be to make IDC transitions in response to the appearance or disappearance of a cue(s) coupled to specific times of day. In evolutionary ecology, such responses to environmental factors are called "adaptive phenotypic plasticity" (Pigliucci et al., 2006). A phenotypic plasticity strategy contrasts from an hourglass timer, in that plasticity sets the duration of IDC stages by stop/go environmental triggers, whereas an hourglass sets the timing of a transition from one IDC stage to the next and all transitions until the IDC is completed occur after set durations. In many cases of adaptive phenotypic plasticity, organisms do not respond

directly to the environmental factor that matters for fitness, but to a proxy that correlates with it. Proxies are particularly useful when the important environmental factor is hard to measure accurately or if the organism needs to prepare in advance of a particular environmental change. For example, Eurasian blue tits (*Cyanistes caeruleus;* a passerine songbird) use a combination of temperature and day length information to predict when to lay eggs such that the caterpillars needed to raise young will be most abundant when the young hatch (Phillimore et al., 2016). Malaria parasites are capable of adaptive phenotypic plasticity, adjusting, for example, investment into gametocytes, and the ratio of males to females, in response to changes in many aspects of the within-host environment in manners that maximise fitness (including, the presence of competing strains, host anemia, and anti-malarial drugs; Reece et al., 2008; Schneider et al., 2018a). Plasticity in IDC traits has also been documented, including quiescence of ring stage *P. falciparum* parasites (Witkowski et al., 2010), changes in the number of merozoites per schizont in calorie restricted (Mancio-Silva et al., 2017) or anemic hosts (Birget et al., 2019), and a longer IDC duration in anemic hosts (Birget et al., 2019). These observations suggest that malaria parasites can regulate aspects of the IDC in response to variable conditions experienced during infections. However, whether these alterations maximise fitness and whether these traits are sensitive to time-of-day are unknown.

**Integrating host-parasite interactions into the IDC**

Early work – mostly theoretical models – assumed that parasites are intrinsically arrhythmic and allow host circadian innate immune responses to impose the IDC schedule upon them (e.g. Kwiatkowski, 1989). Specifically, if different IDC stages vary in how vulnerable they are to a danger that only appears at a certain time of day, a schedule will be enforced on the IDC such that parasites must pass through the vulnerable IDC stage before or after the dangerous window. Early and late IDC stages differ in sensitivity to immune responses and high temperatures associated with fever (Karunaweera et al., 1992; Khoury et al., 2017; Rouzine and McKenzie, 2003), and fever is rhythmic because it is elicited by synchronous schizogony. However, it is hard to reconcile that fever is required to make parasites rhythmic if fever is only elicited by rhythmic parasites; i.e., something else is required to make parasites rhythmic enough to cause a fever rhythm. This difficulty could be overcome if regardless of when schizogony occurs, or the synchrony of parasites, hosts only generate fever at a particular time of day. Experiments by Prior et al., (2018) strongly suggest this is not the case. The time-of-day that cytokines associated with the schizogony peak coincides with when schizogony occurs, regardless of when in the day it occurs. Specifically, if schizogony is delayed by 6 hours (by using mismatched infections to decouple the timing of schizogony with host time-of-day), the cytokine peak is also delayed by 6 hours. Thus, even if these immune responses are more effective against certain IDC stages, they could only increase the synchrony of the IDC, not alter its timing. Put another way, the host fever response can only enforce the schedule that the IDC is already following. Instead, mismatched parasites on the “wrong” IDC schedule return to the “correct” schedule within a few days (Prior et al., 2018).

Host immune responses cannot be solely responsible for imposing the IDC schedule, but do play an indirect role through their interaction with host metabolism (Prior et al., 2018, Hirako et al., 2018). As well as glucose, which is needed as an immediate source of energy for all parasite life stages, completing the IDC (as with any DNA replication cycle) requires a variety of nutrients and resources to progress through cell cycle checkpoints and build cellular machinery. In particular, the later IDC stages require phospholipids for cell membrane formation, purines (especially hypoxanthine) for nucleic acid synthesis, lysophosphatidylcholine (lysoPC) as a source of choline- and fatty-acid-containing products (Brancucci et al., 2017), folate for DNA synthesis (in addition to *de novo* biosynthesis, Hyde, 2005), nicotinamide for NAD+ biosynthesis for glycolysis (Gardner et al., 2002) and amino acids (Babbitt et al., 2012). Some of these resources are scavenged by parasites from digestion of hemoglobin but others are only available from the host’s food. If the parasite is

unable to generate or stockpile an essential resource throughout the day, it may be forced to coordinate development of the more metabolically active (resource hungry) stage(s) with the host's feeding/digestion rhythm. Indeed, the IDC schedule can be coordinated by host feeding (Fig 4a, Prior et al., 2018; Hirako et al., 2018). Specifically, the IDC of *P. chabaudi* is timed such that schizogony is completed towards the end of the host's active phase, when the daily foraging effort comes to an end.

In murine hosts, which are naturally active and foraging at night, schizogony occurs at night, but if hosts are only given access to food in the daytime, the IDC inverts and schizogony occurs in the middle of the day (Fig 4a). Moreover, the day- and night-fed hosts in the experiments conducted by both Prior et al., (2018) and Hirako et al., (2018) were housed under the same light-dark conditions, enabling two hypotheses to be rejected: that parasites themselves are sensitive to light-dark cycles, and that the IDC schedule is governed by rhythms generated by the host's SCN (which tracks the light-dark cycle). Feeding, activity, and metabolism are temporally correlated and cause body temperature to be elevated compared to the rest phase. However, a role for temperature in scheduling the IDC is unlikely; whilst body temperature rhythms are disrupted by daytime feeding, they are not inverted, unlike the timing of the IDC (Prior et al., 2018). Thus, unlike *Trypanosoma brucei,* the host's temperature rhythm is unlikely to set the schedule for the *Plasmodium* IDC, and instead, parasites are sensitive to a peripheral food-related oscillator (Damiola et al., 2000) or simply, the products of digestion appearing in the blood.

Hirako et al., (2018) also identify how inflammatory responses interact with host feeding rhythms to set the IDC schedule (Fig 4b). Malaria infection stimulates TNF-$\alpha$ production which increases the glucose demands of leukocytes and liver cells. Meeting this demand alters glucose metabolism, exacerbating the blood-glucose nadir (causing hypoglycemia) in the host's rest phase, which is when the least metabolically active stages predominate (rings and early trophozoites). Thus, IDC completion occurs when blood glucose levels are elevated by feeding at the beginning of the host's active phase. Further support for the indirect role of pro-inflammatory cytokines and glucose on the IDC schedule comes from observations that in diabetic or TNF-$\alpha$ deficient mice that have no, or attenuated, hypoglycemia, *P. chabaudi* loses synchrony (Hirako et al., 2018).

**Fitness consequences of the IDC schedule across the life cycle**

Scheduling the IDC so that asexual stages proliferate efficiently may entirely explain why malaria parasites have evolved to coordinate the IDC with host feeding. However, early literature on the topic assumed that the fitness benefits of the IDC schedule concern coordinating transmission stage (gametocyte) development with vector (mosquito) activity rhythms, not host rhythms. This notion has precedent across taxonomically diverse parasites. For example, the transmission forms (microfilariae) of several parasitic filarial nematodes only aggregate in the peripheral circulation at the time of day when mosquito vectors forage for blood (Hawking, 1967). Similarly, the cercariae of different subspecies of *Schistosoma* flukes exit their intermediate host at the time of day when they are most likely to locate their next type of host (Lu et al., 2009; Su et al., 2013). How microfilaria and cercariae determine when to migrate/exit are unknown. To transmit to mosquitoes, malaria parasites must undergo a single round of sexual reproduction. Upon ingestion by a blood-feeding mosquito, asexual stages die but gametocytes rapidly undergo gametogenesis and mate. By undergoing schizogony at night, gametocytes were proposed to reach sexual maturity the following night and so, coincide their short window of infectiousness with the time-of-day at which mosquitoes forage for blood (Hawking, 1970). Hawking's hypothesis requires that gametocytes have a short maturation time (before natural variation in gametocyte development rate erodes synchrony), a short lifespan, are not able to alter the developmental duration of gametocytes in response to time-of-day cues, and must use host feeding rhythms as a proxy for vector activity patterns. Subsequent work has shown that not all of these requirements are met.

Most studies have not found evidence for time-of-day variation in the transmission success of *P. falciparum* (Magesa et al., 2000; Karunaweera et al., 1992; Bray et al., 1976; Githeko et al., 1993). These negative results may be due to the long lifespan of *P. falciparum* gametocytes, but these studies did not account for daily rhythms in mosquitoes themselves. If mosquitoes exhibit a daily rhythm in resistance to infection that opposes a rhythm in parasite infectivity, vector and parasite rhythms could cancel each other out. Put another way, if maximum vector resistance coincides with maximum parasite infectivity, and minimum vector resistance coincides with minimum infectivity, the outcome is an intermediate level of transmission at all times of day. Mosquito physiology and behaviors are governed by daily rhythms and many rhythmic processes could affect their susceptibility to malaria infection (Rund et al., 2016; Jones et al., 1967; Das and Dimopoulos, 2008). For example, rhythms in oxidative stress resulting from blood digestion, or rhythms in immune responses could impose time-of-day variation in how well parasites mate, establish midgut infections, replicate, and migrate to the mosquito's salivary glands. A recent study of *P. chabaudi* decoupled time-of-day for both parasites and mosquitoes by using opposite light schedules so they could be investigated separately (Schneider et al., 2018b). Gametocytes are more numerous in the host blood during the daytime but are more infectious at night (more likely to establish midgut infections), regardless of whether the mosquito receives the blood in the day or night. Such time-of-day variation in transmission success translates from a lab-model to a natural host-parasite-vector association: the avian malaria *P. relictum* is also more infectious in the evening (Pigeault et al., 2018). For *P. chabaudi,* rhythms experienced inside vectors matter, but only later in sporogony: both day- and night-time gametocytes achieve higher sporozoite densities in day-biting compared to night-biting mosquitoes (Schneider et al., 2018b).

Taking studies of asexual stages and gametocytes together, it appears that both host and vector rhythms impose selection on the IDC schedule. Mismatch to the host rhythm causes a loss of both asexual stages and gametocytes (O'Donnell et al., 2011; 2013), suggesting that very conveniently for parasites, the same IDC schedule may be beneficial to both asexual stages and gametocytes, or an aspect of host rhythms determines infectiousness. Alternatively, there may be a trade-off in which natural selection prioritizes the timing of either asexual stages or gametocytes at the expense of the other. Testing whether the IDC schedule is a compromise between prioritizing asexual replication or gametocyte infectiousness matters. If there isn't a trade-off, parasites may be unable to alter the IDC schedule and/or the development of gametocytes to keep up with recently reported changes to vector biting rhythms. If so, the evolutionary options for parasites include coercing the host into altering its foraging time, or attracting mosquitoes whenever gametocytes are optimally infective. *T. brucei* provides a proof of principle for parasite manipulation of host circadian rhythms; it shortens period and phase advances the circadian clock of the host in the SCN and several peripheral tissues by ~2 hours during the chronic stages of infection (Rijo-Ferreira et al., 2018). Furthermore, manipulation of host foraging rhythms is proposed for *Potamopyrgus spp.* snails infected with *Microphallus spp.* trematodes (Westwood et al., 2019).

## Remaining mysteries

Recent research has overturned dogma about how and why malaria parasites exhibit timed and synchronized development during the IDC. Yet, there is still much to be discovered about the contributions of the host, parasite, and vector, to establishing and maintaining the IDC schedule, as well as identifying why the IDC schedule matters for parasite fitness (Table 1).

### *Who sets the IDC schedule?*

The balance of evidence suggests that parasites benefit from an IDC schedule aligned with the host feeding rhythm. This suggests the host provides a nutrient or factor required by a

particular IDC stage for a limited period each day, so the stage is set for a combination of host and parasite control of the IDC schedule. Parasites that develop too quickly or too slowly to coincide their “needy” stage with the nutrient’s window of availability will be forced to wait until the nutrient is abundant from the host’s next feeding cycle. If parasites have some control over IDC progression, mistimed parasites could develop more rapidly or more slowly to coincide their “needy” stage with the nutrient’s window of availability (Fig 5a i & ii). Alternatively, parasites could become quiescent until the nutrient is available (Fig 5a iii). In contrast, if parasites have little control over IDC progression, mistimed parasites will likely starve and experience elevated mortality risk. The narrower the nutrient’s window of availability and the more important it is for parasite development, the higher the mortality rate of mistimed parasites, and the faster the IDC is brought into line by the host forcing a schedule onto intrinsically arrhythmic parasites (Fig 5a iv). Note, this is analogous to the mechanism by which rhythmic immune responses are proposed to schedule the IDC (Kwiatkowski, 1989), discussed above. Whether mistimed parasites slow down (Fig 5a i) or speed up (Fig 5a ii) the IDC, become quiescent (Fig 5a iii), or die (Fig 5a iv), will manifest as different patterns for how parasite density changes as the IDC reschedules to match the host rhythm (Fig 5a v).

The scenarios illustrated in Figure 5a are not mutually exclusive, but data from *P. chabaudi* infections initiated with ring stages mistimed by 12 hours to the host rhythm suggest that selective death of certain IDC stages (Fig 5a iv) cannot be the main driver of the IDC schedule. This is because these infections suffer only a minor drop in number and only in the first and/or second IDC (O’Donnell et al., 2013), which is insufficient death to result in rescheduling. Moreover, rescheduling to the host rhythm takes multiple IDCs (approximately 5-7, Fig 5b) which is also inconsistent with quiescence (Fig 5a iii). A more parsimonious explanation is that the small number of parasites most severely affected by being mistimed to an essential resource are killed, but others are able to adjust the duration of their IDC by a few hours each cycle and coordination with the host rhythm is gradually regained. If parasites possess a time-keeping mechanism and use it to schedule the IDC, they may use the essential nutrient as both a resource and a time-of-day cue to align the development of the next cohort to an incoming resource. Alternatively, using a proxy that predicts the appearance of the essential nutrient in advance would allow parasites to adjust IDC progression of the current cohort to capitalize on the appearance of the nutrient. Preparing in advance is a key feature of time-keeping strategies and may be particularly important in mixed-genotype infections because the most competitive genotype will acquire the resource first.

Identifying the essential nutrient(s)/resource(s) would facilitate resolving the contributions of the host and parasite to the IDC schedule. Whether the IDC must coincide with a factor that appears rhythmically in the blood as a product of a host clock-controlled process, or simply as a product of digestion is unclear. Many digestive and metabolic processes do not need clock control to be rhythmic, including glucose homeostasis. Hirako et al., (2018) and Prior et al., (2018) highlight blood-glucose as a promising candidate for both a time-of-day cue and an essential, rhythmic, resource (infected RBCs take up 100 times more glucose than uninfected RBC, Mehta et al., 2005; Fig 4b). Importantly, malaria parasites lack key enzymes involved in gluconeogenesis and so, IDC progression relies on an external source of free glucose. Removal of glucose or inhibitors that block glucose metabolism elicit starvation pathways in *P. falciparum* and parasites die (Babbitt et al., 2012). Clearly the role of the host’s glucose homeostasis is to balance glucose release and glycogen storage to minimize fluctuations in blood-glucose around the clock, but nonetheless, periodic hypoglycemia occurs in different mouse models and humans during acute malaria episodes (Elased and Playfair, 1994, White et al., 1983). Isoleucine is also of note: it is the only amino acid *P. falciparum* cannot scavenge from hemoglobin digestion (Babbitt et al., 2012). Further, the majority of isoleucine uptake is glucose-dependent (Martin and Kirk, 2007), perhaps providing another link between blood glucose levels and the IDC schedule. Whether

the resource parasites need is limiting enough at particular times of day to affect IDC progression is unclear but if it is a host metabolite it likely varies during infections depending on both parasite load and how sick the host is.

Identifying if and how malaria parasites keep time may reveal a novel drug target. Determining the extent to which parasite genes influence the IDC schedule is central to predicting the evolutionary responses of parasites to such a drug. Whilst circadian clocks are generally robust in tracking time despite unexpected perturbations environmental conditions, such robustness may be detrimental to malaria parasites. For example, exclusively using a proxy stemming from the host's light/dark driven circadian clock (such as melatonin; Hotta et al., 2000; Garcia et al., 2001) may not always accurately inform when host feeding occurs because hosts sometimes find food at the start or the end of the active period, and energetically challenged rodents switch from nocturnal to diurnal activity (van der Vinne et al., 2014). Whilst flexibility in the IDC schedule may be maximized by responding to the factors that actually affect parasite fitness, rather than using a proxy, this may trade off against the benefits of anticipation provided by a proxy.

***What explains the evolution of the IDC schedule?***

Whatever the costs and benefits of the IDC schedule for asexual stages and gametocytes, all observations point towards timing being the main selective driver for the evolution of the IDC schedule, with synchronicity being an emergent property (Mideo et al., 2013, Greischar et al., 2014). If parasites benefit from timing their IDC to coincide with an essential resource, it follows that there must be a cost of missing this opportunity. Whilst the modest cost recovered in experiments (O'Donnell et al., 2011; 2013) may seem surprising, even a small advantage over competitors can be favored by natural selection. It is also likely that costs are density dependent. The blood concentration of an essential nutrient may be sufficient even at its nadir, especially in asymptomatic hosts, to support parasites at very low density. If so, asynchronous and scheduled parasites may have equal fitness at the start of infections, but the benefits of timing the IDC schedule to coincide with food intake may become more apparent as density increases. At very high densities, however, synchronized parasites might inadvertently compete with each other and relaxation of the IDC schedule becomes advantageous. These scenarios predict that an IDC schedule is selectively neutral at low densities, beneficial at intermediate densities, and costly at very high densities. Thus, the costs and benefits of synchrony may differ across *P. chabaudi* strains that vary in virulence and also differ between rodent *Plasmodium* species which have a synchronous versus an asynchronous IDC. Why some species (e.g. *P. berghei* and *P. yoelii*) are asynchronous is unclear but they specialize in infecting immature RBC (reticulocytes) which appear in the circulation in a circadian manner and are scarce at the start of infections (Killick-Kendrick and Peters, 1978; Clark and Korst, 1969), so perhaps asynchrony avoids inadvertent competition.

The IDC schedule is also important for transmission because it generates periodicity in infectiousness (Schneider et al., 2018b). There are several non-mutually exclusive explanations for time-of-day variation in the infectivity of gametocytes, two of which could apply to species with long lived gametocytes like *P. falciparum.* First, for species such as *P. chabaudi,* a new cohort of gametocytes is produced every 24 hours (at schizogony), they reach maturation between 24-36 hours old and then have a half-life of approximately 14 hours (Schneider et al., 2018b). Thus, a blood meal taken at night contains gametocytes at different ages compared to a blood meal taken in the daytime. Specifically, day-time blood meals contain more gametocytes but a higher proportion of them are too senesced to be infective (Schneider et al., 2018b). Second, the infectivity of gametocytes might depend on a rhythmic host resource. For example, glutamine and glucose are required for TCA cycle function (MacRae et al., 2013). Third, in principle, rhythms in innate host immune responses could clear or sterilize gametocytes at a particular time of day, either in the host's blood or

the vector's blood meal. The latter two hypotheses might explain why mistimed parasites suffer a 50% loss of gametocytes (O'Donnell et al., 2011).

Mosquito rhythms add to the complexity of timing for transmission. That their role manifests late in sporogony (Schneider et al., 2018b) suggests that the vector's feeding time-of-day has long lasting effects on parasites, potentially analogous to the late-acting effects of time-of-day of infection by *Trichuris muris* on immune development (Hopwood et al., 2018). However, at the epidemiological scale, transmission is not simply a product of the prevalence or density of parasites within infected mosquitoes, but also the availability of biting mosquitoes, which is governed by mosquito activity rhythms (Rund et al., 2012; Rund et al., 2016). Deconstructing the contributions of gametocyte rhythms, mosquito rhythms, host rhythms, and their interactions, to transmission is pertinent due to reports that mosquito populations are changing the time of day they forage for blood (to evade insecticide-treated bed nets). There is the potential for high complexity if rhythmic blood components (such as amino acid composition, glucose, and immune factors) interact with rhythms in mosquito immune responses or metabolic processes to affect parasite development during sporogony (Rund et al., 2016). Rhythms in host blood components do not affect transmission of the asynchronous species *P. berghei* (O'Donnell et al., 2019), but a synchronous parasite may potentiate rhythms in host blood components.

**Conclusions: from mice to men?**

Most insights into parasite rhythms have come from animal model systems. Whether the rules for parasites with 24-hour IDCs can be applied to the human parasites with 48- and 72-hour IDCs is yet to be determined. Furthermore, whether relevant rhythms in nocturnal murine hosts are simply inverted compared to those in diurnal humans is unclear. Whilst working with human parasites is challenging, their long IDCs may offer the opportunity to differentiate between genes involved in responding to host circadian rhythms from genes that are rhythmic simply as a consequence of IDC progression. Answers to these questions are urgently needed given reports that dormancy during the IDC helps parasites tolerate antimalarial drugs and insecticide treated bed nets are causing mosquitoes to change the time of day they blood-feed. IDC-disrupting drugs might reduce disease severity and transmission potential. Furthermore, if the optimal schedules for asexual replication and gametocyte development differ, such drugs should be robust to parasite counter-evolution. This is because a trade-off is imposed upon parasites such that parasites cannot alter the IDC in a manner that benefits asexual replication without compromising transmission, and vice-versa.

**Acknowledgments**

KP and SR are supported by the Wellcome (202769/Z/16/Z) and the Royal Society (UF110155; NF140517). RG, PA and IH are supported by NIH (R01-NS098747-04, R01-AI079293-10, R21-AI131632-02, 2U19-AI089681-08), FAPESP-2016/23618-8 and INCT-Vacinas (465293/2014-0). FRF is an Associate in the Howard Hughes Medical Institute. DW is supported by NIH R21 NS103180.

**Declaration of Interests**

The authors declare no competing interests.

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**Table 1. The challenges ahead.** Integrating observations regarding the timing and synchrony of the IDC schedule into an evolutionary ecology framework raise key outstanding questions. Answering these questions will reveal to what extent traits belonging to hosts/vectors and parasites regulate the IDC schedule, and establish the fitness costs and benefits of the IDC schedule to all parties involved.

| Topic | Key questions |
|---|---|
| **Host feeding rhythm** | • Which rhythm(s), associated with meal timing, schedule the IDC (Prior et al., 2018; Hirako et al., 2018)?<br>• Are nutrients / products of digestion directly responsible, or are other food-entrainable host-clock-controlled processes involved? |
| **Rhythms in inflammation** | • How do rhythms in TNF-α and food intake interact to schedule the IDC (Hirako et al., 2018)?<br>• Is the IDC rhythmic in new infections of semi-immune hosts who mount acquired, rather than innate, responses (Reece and Prior, 2018)? |
| **Control of the IDC schedule** | • Do parasites actively control the synchrony and timing of the IDC to coordinate themselves to host feeding rhythms?<br>• Do gene expression patterns and pathways among parasite species with IDCs of differing durations allow signatures of time-keeping to be differentiated from developmentally regulated processes? |
| **Exo-erythrocytic development** | • Do liver-derived merozoites invade RBCs in synchrony with host feeding rhythms, or does the IDC only become aligned to host feeding rhythms once parasites are in the blood? |
| **Quantifying IDC parameters** | • Can within-host models overcome the biases that sequestration, death, and re-invasion by progeny merozoites may introduce to estimates of synchronicity and timing (Greischar et al., 2019)? |
| **Costs/benefits of IDC rhythms** | • If parasites schedule the IDC to best exploit a host resource, is this unnecessary when they are at low density, and is synchrony costly at high density due to closely related parasites competing for the resource?<br>• Do the costs and benefits of synchrony alter when hosts become anorexic during the acute phase of infection? |

| | |
|---|---|
| **Rhythms in gametocytes** | • Do changes in the proportion of parasites committing to becoming gametocytes (conversion rate, Schneider et al., 2018a) or vulnerability to immune factors (Long et al., 2008) account for the reduction in gametocytes that occurs when parasites are out-of-synch with host rhythms (O'Donnell et al., 2011)?<br>• Do host rhythms regulate infectivity to vectors?<br>• Can gametocytes adjust their developmental rate to achieve synchrony with host rhythms? |
| **Rhythms in transmission** | • Why does blood-feeding mosquitoes in their day-time (rest phase) enhance infection success (Schneider et al., 2018b)?<br>• How does the circadian physiology of vectors interact with daily environmental temperature rhythms to shape transmission?<br>• Is the host more susceptible to infection by incoming parasites at certain times of day? |

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Fig 1

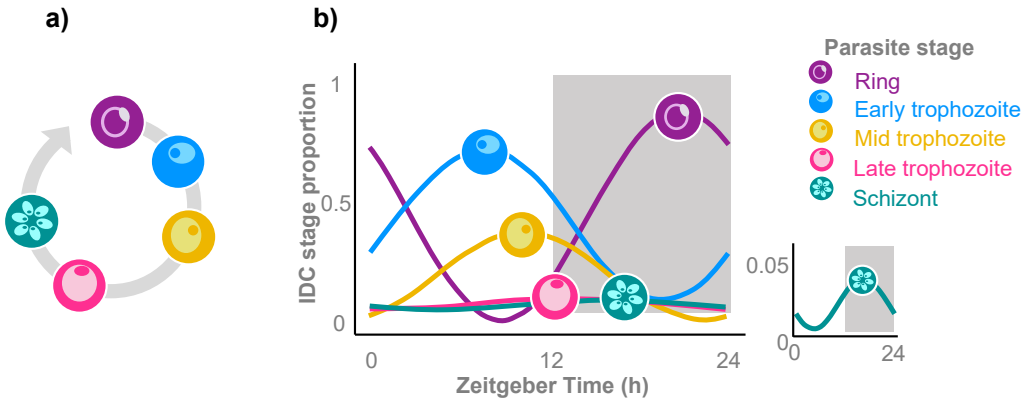


**Figure 1. Periodicity in malaria parasites**

**a)** Following transmission from a mosquito, malaria parasites replicate in the liver before invading red blood cells and undergo successive cycles of asexual replication (the intra-erythrocytic development cycle, IDC). The IDC begins when a merozoite invades an RBC, which then becomes a ring stage parasite (purple), before developing through several developmental stages of the trophozoite stage (blue, orange, pink), and finally becoming a mature schizont (teal) which ruptures to release merozoites to initiate the next IDC. Each stage within the IDC has different roles and requirements from the host. The ring stage remodels the RBC and has no specific requirements, trophozoites undergo DNA replication and need amino acids, glucose, purines, folates and lysophosphatidylcholine, while schizonts complete cell division and require phospholipids for membrane production. **b)** Throughout the rodent host's circadian cycle (white bar = day, grey bar = night), each IDC stage develops in sequence. Later stages (mid trophozoites to schizonts) sequester out of circulation in capillaries and organs and have very low abundance (and therefore dampened rhythms) in circulation. However, zooming in, for example, on schizonts (inset) reveals rhythmicity, with their peak occurring just before their ring stage progeny appear. Rhythms shown are model fits from data in Prior et al., (unpublished). Zeitgeber Time refers to the number of hours since lights on/dawn.

Fig 2

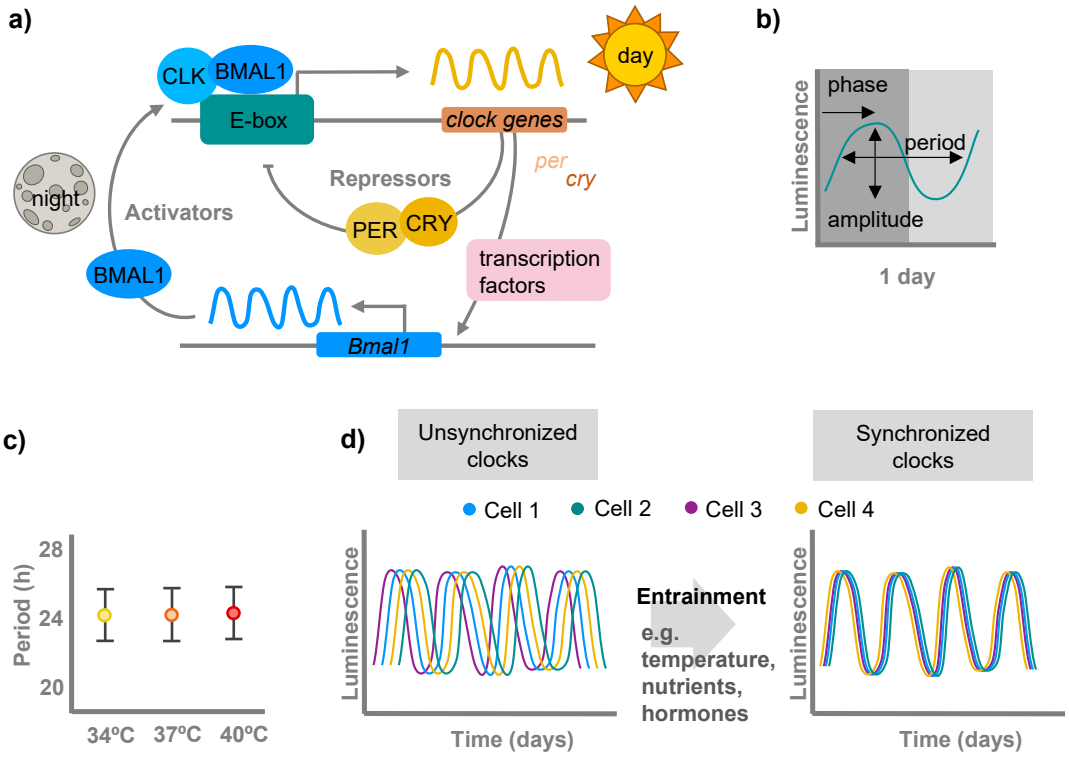


**Figure 2. Chronobiology concepts**

**a)** Core molecular components of the mammalian TTFL (Transcription-Translation Feedback Loop) clock consist of the proteins CLOCK (CLK), BMAL1, PERIOD1 and PERIOD2 (PER) and CRYPTOCHROME1 and CRYPTOCHROME2 (CRY). These components, with the help of other transcription factors and proteins, cycle through transcription, translation and degradation cycles with a period of ~24h, instructing downstream target genes to orchestrate rhythms in physiology and behavior (Reppert and Weaver, 2002). Figure modified from Rijo-Ferreira et al., (2017a). **b)** Circadian clocks have a built-in ability to anticipate daily environmental changes. The activities of circadian clocks can be assessed by directly measuring clock-controlled gene expression (either clock genes or those downstream), commonly done using a luminescent reporter (Yamazaki et al., 2000). Circadian rhythms are characterized by their “period” (of around 24 hours), “phase” (time-of-day the rhythm reaches a point of biological relevance), and “amplitude” (difference between peak and trough values). These features persist due to clock-control even in the absence of environmental rhythms (“free running”); here the subject is kept in constant darkness and the light and dark grey bars illustrate “subjective” day and night. **c)** Circadian clocks are also “temperature compensated” and so, maintain the same period across a gradient of environmental temperatures (Pittendrigh, 1960). **d)** Circadian clocks usually operate in rhythmic environments and all clocks require a “Zeitgeber” or time cue to “entrain”. When cultured without external stimuli for many days, clocks retain individual oscillations but usually become desynchronized from each other. Synchronization is achieved by entrainment. The features illustrated (**b-d**) ensure that clock oscillators balance being robust to perturbation whilst being flexible enough to keep up with (for example) changing photoperiod across seasons.

Fig 3

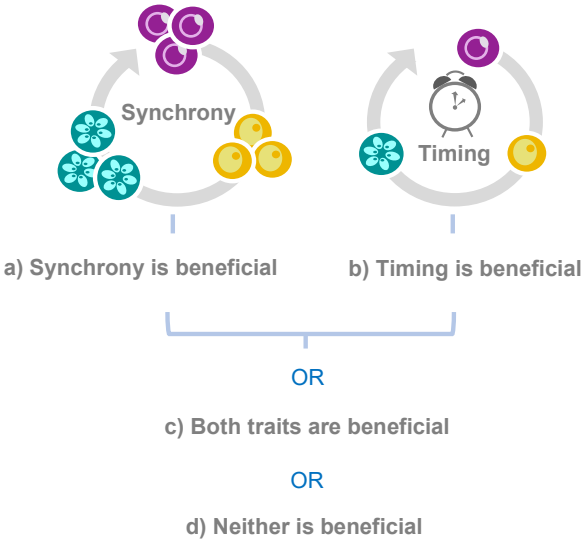


**Figure 3. Evolutionary explanations for the IDC schedule**

Understanding the evolution of the IDC schedule requires explaining why both synchrony and timing occur. Selection could favor both traits, one trait, or neither of the traits, as illustrated by these scenarios. **a)** Synchrony is beneficial: Synchronous development enhances fitness, but timing does not. Timing may vary or be used as the mechanism to achieve synchrony. **b)** Timing is beneficial: An IDC transition at a particular time of day enhances fitness, so synchrony occurs as a by-product of selection for timing. For example, timing may be beneficial to parasites simply because it enables them to maximally exploit a rhythmic resource inside the host. In the scenarios in which one trait is favored, the other could be costly (*i.e.,* a constraint) but the benefits of the useful trait outweigh these costs. The alternative is that both **(c)** or neither **(d)** trait are beneficial to parasites. **c)** Both traits are beneficial: Fitness is enhanced by each trait for different reasons so both traits are independently favored by natural selection. For example, synchrony may provide safety in numbers against a harmful host factor, and if this harmful factor is rhythmic and IDC stages vary in their vulnerability to it, then timing is also beneficial. **d)** Neither are beneficial: Synchrony and timing could be an unavoidable by-product of a different trait that confers sufficient fitness benefits to offset the costs of synchrony and timing. Alternatively, parasites may have no control of the IDC schedule and synchrony and timing are forced upon the parasite by host rhythms (regardless of whether this is beneficial to the host or not).

Fig 4

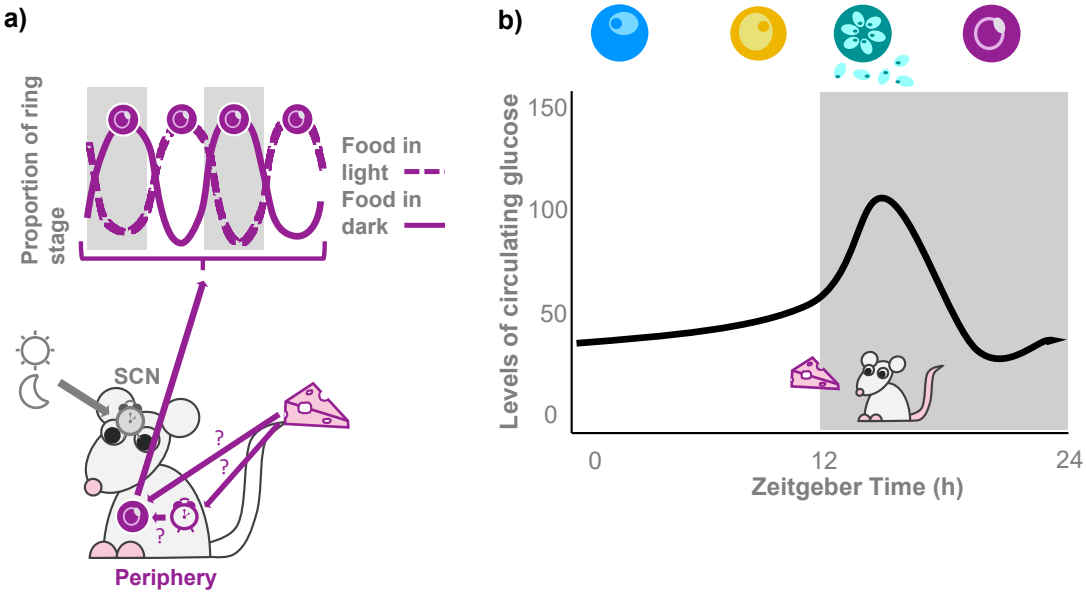


**Figure 4. What sets the timing of the IDC?**
**a)** In *P. chabaudi,* the timing of the IDC can be driven by the feeding rhythm of the host. Mammalian hosts have a clock in the suprachaismatic nucleus (SCN) and peripheral clocks in other organs and tissues. The SCN clock uses light as its primary time cue and the timing of peripheral clocks is most sensitive to cues from food/metabolism. The cartoon illustrates the results of Prior et al., (2018) and Hirako et al., (2018) in which schizogony peaks during the period in which hosts are provided with food, irrespective of the timing of the hosts SCN clock. The precise mechanism by which parasite rhythms result from the timing of food intake is unclear, as indicated by the question marks (?). **b)** The generation of synchrony and timing of the parasite IDC is still not fully understood. Parasites develop through the IDC, from being relatively metabolically inactive to undergoing DNA replication and cell division, as hosts move from the rest to the active phase of their daily rhythm. Host activity corresponds with food intake, which elevates blood glucose concentration. As infections progress, pathogen-associated molecular patterns (PAMPs) activate immune cells via TNF-α which alters their energy metabolism. This, in concert with the production of inflammatory cytokines stimulating glucose uptake from the blood (by for example, the liver), exacerbate the host's daily rhythm in blood glucose. The resulting daily window of hypoglycaemia corresponds with metabolically inactive stages in the IDC, and hyperglycaemia corresponds with IDC completion, generating both synchrony and timing of the IDC (Hirako et al., 2018, Prior et al., 2018, Reece and Prior, 2018).

Fig 5

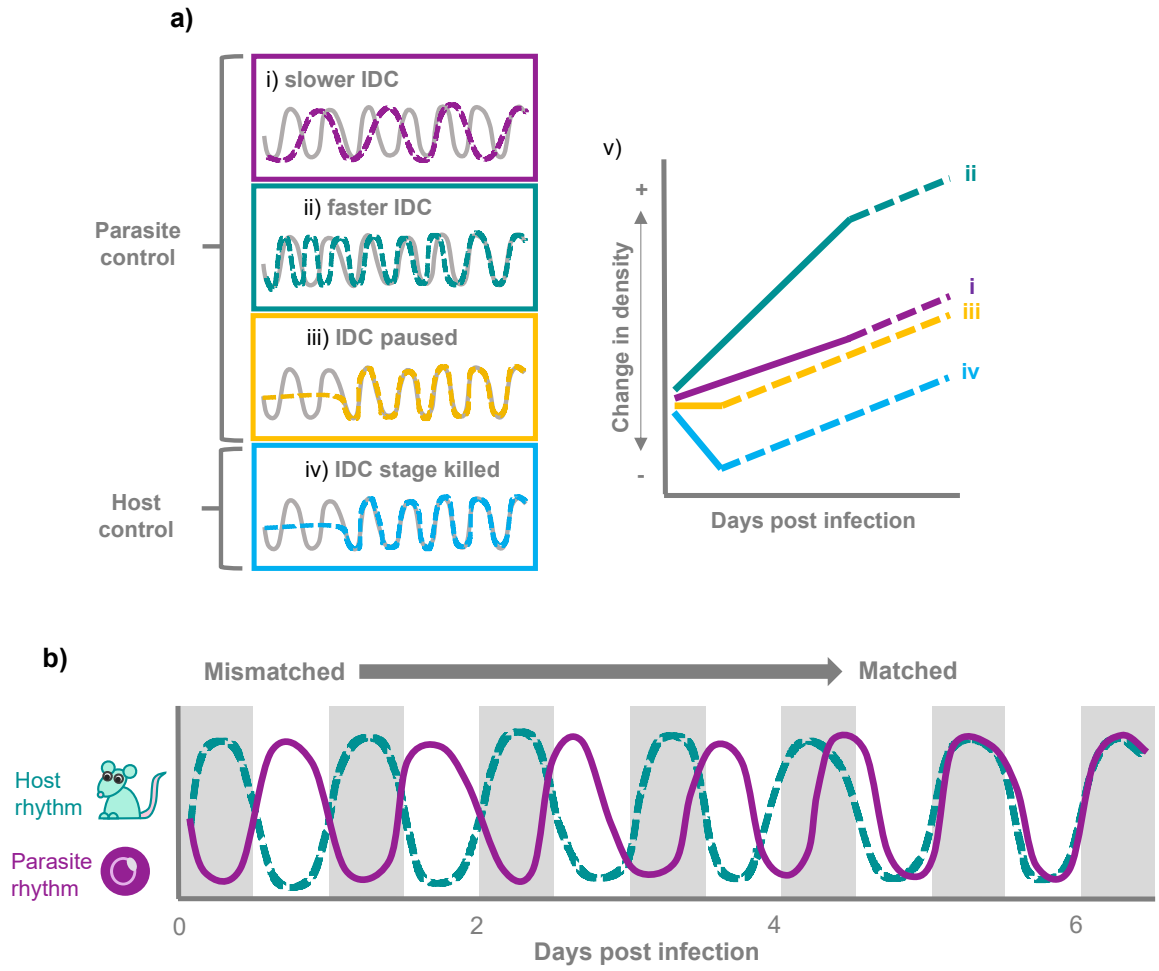


**Figure 5. Who controls the IDC schedule?**
**a)** To what extent the IDC schedule is organized by the parasite versus forced by the host is unclear. The possible scenarios (i – iv) are illustrated by considering how *P. chabaudi* parasites (dashed line) whose IDC is mismatched to the host feeding rhythm become rescheduled to follow the same pattern as control (i.e., matched; grey lines) infections. If parasites control the IDC schedule, they can (i) slow or (ii) speed up the IDC (within constraints set by the minimum and maximum possible duration of each IDC stage), or (iii) become quiescent until an IDC stage encounters the correct time-of-day to develop. Alternatively, (iv) if the parasites are passive to being scheduled solely by the host, mismatched parasites will be forced to stop at some point in the IDC. Depending on how long parasites can remain quiescent and the degree of stress imposed on parasites by being forced to stop developing, scenario (iv) is hard to differentiate from scenario (iii). But, if parasites whose development is stopped die, then examining densities during rescheduling (v) can differentiate the scenarios. (v) Assuming burst size, invasion rate etc. do not differ between the scenarios, and all infections replicate at the same rate once rescheduled (i.e., slopes are parallel), comparing densities during (solid lines) and after rescheduling (dashed lines), differentiates the scenarios. Observations that the IDC remains mismatched for a few cycles suggests scenarios iii and iv are unlikely. **b)** If the *P. chabaudi* IDC is mismatched to the host rhythm, the IDC returns to synchrony with the host rhythm within 5-7 cycles. When infections are initiated using ring stages transferred from donor hosts to recipient hosts on an opposite light schedule (O'Donnell et al., 2011), the recipient host rhythm (dashed green line) remains unaltered but the parasite IDC (purple line) becomes rescheduled to the

recipient host rhythm, whilst remaining synchronous (Prior et al., 2018). Dark bars indicate night and light bars indicate day.