Synchrony between daily rhythms of malaria parasites and hosts is driven by an essential amino acid

Kimberley F. Prior[1,2], Benita Middleton[3], Alíz T.Y. Owolabi[1,2], Mary L. Westwood[1,2], Jacob Holland[1,2], Aidan J. O'Donnell[1,2], Mike Blackman[4], Debra J. Skene[3], Sarah E. Reece[1,2]

Institutes of Evolutionary Biology[1] and Immunity & Infection Research[2], University of Edinburgh, UK; Faculty of Health and Medical Sciences, University of Surrey[3], UK; Francis Crick Institute[4], UK.

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Rapid asexual replication of malaria parasites is responsible for the severity of disease symptoms and fuels investment in transmission. That malaria parasite species coordinate their cycles of replication with daily rhythms of their host was discovered in the Hippocratic era, but how and why this occurs is enigmatic. Here, we demonstrate that the parasite's schedule for asexual replication can be orchestrated by a metabolite provided to the parasite from the host's rhythmic intake of food. We search for nutrients with daily rhythms in the blood that match the rhythms in both host feeding and parasite development. Only one metabolite - the amino acid isoleucine – fits these criteria, is essential, and must be scavenged from blood. Further, perturbing the timing of isoleucine provision and withdrawal, reveals that isoleucine fits the criteria for time-cue to schedule and synchronise asexual replication. Identifying how and why parasites keep time opens avenues for interventions.

Circadian rhythms are assumed to have evolved to coordinate organisms' activities with daily rhythms in the environment (Ouyang et al 1998, Spoelstra et al 2016, Hozer et al 2021). The value of organising sleep/wake cycles according to whether it is light or dark, whether predators or prey are active, *etc*., is clear, but how parasites cope with rhythmicity in the within-host environment has been neglected (Martinez-Bakker & Helm 2015, Reece et al 2017, Westwood et al 2019). Many of the processes underlying interactions between hosts and parasites have a circadian basis, yet why these rhythms exist and what their consequences are for hosts and parasites remain mysterious. For example, parasites are confronted with rhythms in host behaviours and physiologies, including immune responses and metabolism. Thus, some host rhythms offer opportunities for parasites to exploit, whilst others impose constraints parasites must cope with. Host daily rhythms have such an impact on parasite fitness that some parasites use their own circadian clocks to control metabolism (Rijo-Ferreira et al 2017), and virulence (Hevia et al 2015). Host rhythms also have fitness consequences for malaria (*Plasmodium*) parasites (O'Donnell et al 2011, 2013), whose rhythmicity in development during blood-stage replication is aligned with the timing of host feeding-fasting cycles (Hirako et al 2018, Prior et al 2018, O'Donnell et al 2020).

Explaining the timing and synchrony exhibited by malaria parasites during successive cycles of blood-stage replication has been a puzzle since the Hippocratic Era, when the duration of these cycles (24-, 48, or 72-hours, depending on the *Plasmodium spp.*) were used to diagnose malaria infection (Garcia et al 2001, Mideo et al 2013). Blood-stage replication is orchestrated by the intraerythrocytic development cycle (IDC), which is characterised by parasite invasion of red blood cells (RBC), growth, division, and finally bursting RBC to release the next cohort of asexually replicating parasites. Given that asexual replication is responsible for the disease symptoms of malaria and fuels the production of transmission forms, explaining how and why the vast majority of *Plasmodium* species progress though the IDC in synchrony, and why transitions between these stages occur at particular times of day, may unlock novel interventions and improve the efficacy of existing treatments. However, such endeavours rely on identifying the precise host rhythm(s) that parasites align to and explaining why this matters for their fitness (O'Donnell et al 2011, Prior et al 2020).

Malaria parasites are able to keep time (Rijo-Ferreira et al 2020, Subhudhi et al 2020) and organise their IDC schedule to coordinate with an aspect of host feeding-fasting rhythms (Hirako et al 2018, Prior et al 2018), but not the act of eating itself (Rijo-Ferreira et al 2020), or metabolic processes directly controlled by canonical host circadian clocks (O'Donnell et al 2019). Coordination with host feeding-fasting rhythms may allow parasites to couple the nutritional needs of each IDC stage with circadian fluctuations in the concentrations of nutrients in the blood (Prior et al 2020). Whilst parasites are able to scavenge most amino acids from haemoglobin digestion, and can biosynthesise some metabolites themselves, other nutrients are essential and must be taken up from the RBCs or blood plasma. Haemoglobin digestion and biosysnthesis can occur at any time-of-day but essential nutrients that both host and parasite must acquire from the host's food (including glucose and the amino acid, isoleucine) follow circadian rhythms in the blood (Skene et al 2018). Hosts forage during their active phase and fast during the rest phase, generating peaks in the blood concentrations of metabolites shortly after the onset of feeding, which reach their lowest point (nadir) during the fasting phase.

Nutritional needs vary across IDC stages, with for example, requirements for glycerophospholipids and amino acids to fuel biogenesis, increasing as parasites progress through the IDC, and the release of parasite progeny at the end of the IDC consumes a lot of glucose (Olszewski et al 2009, Déchamps et al 2010, Fig. 1a). Whether essential nutrients are available around-the-clock at sufficient concentrations to satisfy the needs of parasites is unknown, but in culture, IDC progression is affected by nutrient limitation. For example, parasites experiencing glucose limitation mount starvation responses (Daily et al 2007) and isoleucine starvation induces dormancy (Babbitt et al 2012). Moreover, the nadir of blood glucose rhythms in the rest phase can be exacerbated by malaria infection (Hirako et al 2018). Thus, to avoid the problems of temporal resource limitation, parasites may match transitions between IDC stages to rhythmicity in the availability of essential nutrients in the blood. Further, parasites could use an essential nutrient itself as a cue to coordinate with the timing of host feeding-fasting rhythms, and/or allow the host to impose the IDC schedule by, for example, selectively killing IDC stages that are not "on time". By getting the timing of the IDC schedule right, parasites not only maximise asexual replication (which underpins within-host survival) (O'Donnell et al 2011, 2013), but also benefit from coordinating the maturation of sexual transmission stages with the time-of-day their vectors forage for blood (Pigeault et al 2018, Schneider et al 2018).

Results

We integrate evolutionary ecology and parasitology with chronobiology to, firstly, undertake a hypothesis-driven screen of several metabolite classes and glucose to identify those with daily fluctuations in the blood of malaria-infected hosts. Secondly, we determine which metabolites follow rhythms set by the timing of host feeding-fasting perturbations plus match the timing of the IDC schedule. We find that fluctuations in blood glucose concentrations and almost all metabolites cannot explain the IDC schedule, but our screens reveal a single candidate; the amino acid, isoleucine. Thirdly, we test how the timing of isoleucine provision and withdrawal affects IDC progression in the manner expected if parasites use it as a time-of-day cue to schedule their IDC. We capitalise on the rodent malaria *P. chabaudi* model system in which *in vivo* experiments exploit ecologically relevant host-parasite interactions and short-term *in vitro* studies allow within-host ecology to be probed in-depth.

Metabolites that associate with host feeding rhythms and the IDC schedule. To identify metabolites whose rhythms correspond to the timing of host feeding and the IDC schedule, we compared four groups of malaria infections in mice that were either wild type (WT) C57BL/6J strain or Per1/2-null (Period 1 and Period 2) circadian clock-disrupted mice. Per1/2-null mice have an impaired canonical clock (Transcription Translation Feedback Loop, TTFL) and exhibit no circadian rhythms in physiology or behaviour when kept in constant darkness (Bae et al 2001, Maywood et al 2014). We generated 3 different groups of hosts whose feeding-fasting rhythms differed with respect to the light:dark cycle and whether they had an intact TTFL clock, and a 4th group of hosts which lacked both feeding rhythms and an intact TTFL clock (Fig. 1b). All infections were sampled every 2 hours from day 5 post infection (when infections are at a low parasitaemia, ~10%, to minimise the contribution of parasite molecules to the dataset, Olszewski et al 2009) for 26 hours. We hypothesised that a time-cue/time-dependent resource must vary or have rhythmicity across 24 hours, with a peak concentration that associates with the timing of host feeding-fasting as well as the same IDC stage, across the 3 treatment groups with rhythmic feeding plus a rhythmic IDC, yet be arrhythmic in the 4th group (Fig. 1b). Thus, we identified candidate metabolites by intersecting rhythmic metabolites in the treatment groups (Fig. 1c).

IDC schedules followed the expected patterns for each treatment group (Fig. 2, and O'Donnell et al 2019). Specifically, parasites displayed opposite patterns in dark (i.e. night, DF, n=18) and light (i.e. day, LF, n=17) fed wild type (WT) mice because their feeding-fasting rhythms are inverted (Fig. 2, Supp Table 1). Parasites in Per1/2-null mice (kept in constant darkness) were fed during a time-restricted 12 h window (TRF mice, n=17) that coincided with when LF mice were fed, followed the same IDC schedule as parasites in LF mice. Finally, parasites in Per1/2-null mice (kept in constant darkness) with constant access to food (ALF mice, n=16), exhibited dampened IDC rhythms because their hosts have no feeding-fasting rhythm (O'Donnell et al 2019). Across the entire data set, 110 metabolites varied during the 26 hour sampling window (101 in DF, 91 in LF, 50 in TRF and 1 in ALF; Supp Table 3). That only 1 metabolite (lysoPC a C20:3) exhibited a rhythm in ALF hosts demonstrates that host TTFL clocks and timed feeding are required to generate metabolite rhythms (Fig. 3a , Reinke and Asher 2019). Further, that approx. half of the metabolites rhythmic in hosts with TTFL clocks (DF and LF) were also rhythmic in TRF hosts reveals that feeding rhythms alone can maintain metabolic rhythms during infection (Fig. 3a). Of all metabolites, only 42 were rhythmic in all 3 groups of infections with both feeding-fasting and IDC rhythms (i.e. the red area in Fig. 1c), consisting of 3 acylcarnitines, 11 amino acids, 9 biogenic amines and 19 glycerophospholipids (Fig. 3a). Next, we identified 33 metabolites that exhibited a peak phase (timing) that corresponded to the timing of the host's feeding-fasting rhythm and the parasite's IDC (Fig. 3b; metabolites clustering in the blue and purple areas).

Glucose does not associate with the IDC schedule. Previous work implicates blood glucose rhythms as responsible for the IDC schedule (Hirako et al 2018, Prior et al 2018) but glucose could not be measured by the UPLC/MS-MS

metabolomics platform. Therefore, we set up an additional set of DF, LF, TRF and ALF infections (n=5/group), which followed the same feeding-fasting and IDC schedules as infections in the metabolomics screen, Fig. 2, Supp Table 1), to quantify blood-glucose rhythms. Mean blood-glucose concentration differed between the groups, being higher in DF and TRF mice (DF=8.55±0.14 mmol/L, TRF=8.59±0.13 mmol/L) than in LF and ALF mice (LF=7.90±0.14 mmol/L, ALF=7.68±0.11 mmol/L). We found two competitive models including only time of day (ZT/h) or both time-of-day and treatment (DF, LF, TRF and ALF) as main effects, that can explain patterns of glucose concentration (Fig 4, Supp Table 4). Specifically, glucose concentration varied throughout the day in DF mice but much less in LF mice, and glucose was invariant in both groups of Per1/2-null mice (TRF and ALF) (Supp Table 4). The rhythmic IDC schedule in the DF, LF and TRF groups but the lack of significant rhythmicity in blood glucose in the TRF (and possibly LF) infections demonstrates that glucose does not explain the connection between feeding rhythms and the IDC schedule.

Linking metabolites to the IDC schedule. Having ruled out a role for blood glucose, we explored whether the metabolites from our screen could explain why the IDC schedule follows feeding-fasting rhythms. The metabolites whose peak associated with both feeding and late IDC stages, are alanine, asparagine, isoleucine, leucine, methionine, phenylalanine, proline, threonine, valine, methionine-sulfoxide and serotonin. Whereas 20 acylcarnitines and glycerophospholipids (with the exception of 2 biogenic amines) associated with fasting and early IDC stages (ADMA, SDMA, C14.1, C16, C18.1, lysoPCaC16:1, lysoPCaC18:1, lysoPCaC18:2, PCaaC32:1, PCaaC34:4, PCaaC38:3, PCaaC38:4, PCaaC38:5, PCaaC38:6, PCaaC40:4, PCaaC40:5, PCaeC34:3, PCaeC38:0, PCaeC38:3 and PCaeC42:1. See Supp Tables 5, 6). Next, we asked if these metabolites could schedule the IDC, either by being sufficiently limiting at a certain time-of-day to enforce a rhythm on the IDC, and/or by acting as a time-of-day cue (or Zeitgeber) for the parasite to organise its IDC with a "just-in-time" strategy (or a circadian clock). Each of these scenarios require several non-mutually exclusive criteria to be met. First, for the IDC schedule to be enforced by rhythmicity in the availability of a metabolite, it requires that the parasite cannot overcome resource limitation by synthesising the metabolite itself. Second, if parasites use a metabolite as a time-of-day cue/Zeitgeber to schedule the IDC themselves, the cue's peak timing may either coincide with the IDC stage(s) that most needs it, or parasites may detect a cue that occurs in advance of the important metabolite appearing, to enable it to prepare for its arrival (i.e. analogous to anticipation). Third, for a metabolite to be a reliable time signal, it should be something the parasite cannot synthesise to prevent it having to differentiate between endogenous and exogenous information, and avoid the risk of mistakenly responding to an endogenous signal. Of the 33 metabolites on the shortlist, only isoleucine fulfils these criteria (Table 1), suggesting that both hosts and parasites are reliant on the host's food to acquire isoleucine.

Timing and completion of the parasite IDC depends on the availability of isoleucine. That malaria parasites rely on exogenous isoleucine as a both a resource and time-cue to complete the IDC is supported by previous observations. First, when the human malaria *P. falciparum* is deprived of isoleucine, parasites they undergo delayed cell cycle progression (Liu et al 2006) yet are able to recover even after a longer period of starvation (~4 IDCs) (Babbitt et al 2012). Second, in contrast to isoleucine, if other amino acids are removed from culture media, parasites switch to scavenging them from haemoglobin with minor effects on IDC completion (Babbitt et al 2012). Third, not only is isoleucine crucial for the development of P. *knowlesi* in culture, parasites only incorporate isoleucine up until the point that schizogony starts (Polet 1968, Polet 1969, Butcher and Cohen 1971, Sherman 1979). Fourth, isoleucine is one of few amino acids that exhibits a daily rhythm in the blood of mice and humans. Specifically, isoleucine was inverted with a 12 h shift in simulated day and night shift work (a phase difference of 11:49 ± 02:10 h between the day and night shift conditions) and follows the timing of food intake (Skene et al 2018). Therefore, our next experiments tested if an exogenous supply of isoleucine is capable of scheduling the IDC.

We carried out two experiments in parallel to quantify how *P. chabaudi's* IDC progression is affected when deprived of isoleucine, and whether the IDC is then completed (defined as the proportion of parasites that reach the schizont stage, Supp Fig 1) when isoleucine is restored. Isoleucine cannot be perturbed in vivo without causing confounding off-target effects on the host or interference by host homeostasis so we carried these experiments *in vitro*. First, parasites cultured in the absence of isoleucine (n=32 cultures from the blood of 8 mice, which were split equally across both treatments) develop extremely slowly with approx. 3-fold fewer completing the IDC compared to parasites with isoleucine (50 mg/L, which is the same concentration as standard culture media; RPMI 1640) (n=32 cultures) (Fig. 6a). The best fitting model contained only "treatment" (parasites cultured with or without isoleucine) as a main effect (ΔAICc=0, Supp Table 7-A). The reduction in schizonts in isoleucine-free conditions was not due to a higher death rate because the density of parasites remains constant during the experiment and did not differ between the treatments (Fig. 6b) (best fitting model is the null model ΔAICc=0, Supp Table 7-B). Further, incorporating either "treatment" or "hours in culture" into the model did not improve the model fit (treatment: ΔAICc=4.16, hours in culture: ΔAICc=5.50, Supp Table 7-B).

A substantial slowing of IDC progression in the absence of isoleucine is consistent with observations of *P. falciparum* (Liu et al 2006, Babbitt et al 2012, McLean and Jacobs-Lorena 2020) and further supported by our second experiment which revealed that development is completed when isoleucine deprivation ends. Parasites (~10^7 per culture) were

added to isoleucine-free media and incubated for 7, 14, or 18 hours, after which isoleucine (50 mg/L) was added to their cultures (n=16 cultures per treatment). Parasites completed development when isoleucine became available, regardless of the duration of deprivation (7, 14, or 18 hours), with the best fitting model containing main effects of "treatment" and "hours since isoleucine added" (ΔAICc=0, Supp Table 7-C). Importantly, including the interaction did not improve the model fit (ΔAICc=13.65, Supp Table 7-C), demonstrating that IDC completion proceeds at the same rate despite different durations of isoleucine starvation. Specifically, the rate of IDC completion in the 6-9 hours following isoleucine addition was approximately 50% for all groups (Fig. 6c). Again, these completion rates were not driven by higher death rates in cultures deprived of isoleucine for the longest time period because the model incorporating "hours since isoleucine" was competitive with the null model (ΔAICc=0.56, Fig. 6d, Supp Table 7-D), revealing parasites were still viable, even after 18 hours in culture. Furthermore, cultures deprived the longest achieved the most schizonts (18 hours, mean±SEM: $1.61\times10^6 \pm 0.20$ Fig. 6c), while the least schizonts were observed in cultures deprived for the shortest period (7 hours, mean±SEM: $0.83\times10^6 \pm 0.14$ Fig. 6c). The variation in the intercepts of Fig. 6c (<1% schizonts after 7 hours, 3% after 14 hours and 5% after 18 hours deprivation) is likely explained by the 18 hour deprivation cultures accumulating a higher proportion of schizonts at the time of isoleucine provision simply as a product of developing very slowly during a longer window of deprivation (in line with Lui et al 2006 and Babbitt et al 2012).

Discussion

Our large-scale metabolomics screening experiment (Figs. 3-5, Table 1) and follow-up experiments (Fig. 6), demonstrate that isoleucine is sufficient to explain how malaria parasites schedule their IDC in line with host feeding-fasting rhythms. Our findings are not simply a case of revealing that parasites cannot develop when resource limited. When deprived of other essential resources, *P. falciparum* displays stress responses at the transcriptional level yet when deprived of isoleucine its normal transcriptional pattern persists, albeit very slowly (Babbitt et al 2012), suggesting it is waiting for a signal to proceed (a "gate"). Further, that development restarts with no apparent ill-effects when isoleucine is detected, and that parasites cannot create isoleucine stores, or generate it endogenously, are all hallmarks of a cue-response system. Thus, we propose that isoleucine is used, at least in part, as information used by parasites to schedule the IDC.

Glucose has previously been suggested as a time-cue or scheduling force for the IDC (Hirako et al 2018, Prior et al 2018). Our data do not support this, but, glucose may be indirectly involved. Parasites that are glucose deprived fail to concentrate isoleucine (Martin and Kirk 2007), likely due to a lack of glycolysis and ATP production needed to operate isoleucine transporters. High concentrations of isoleucine in the blood are also associated with uptake of glucose by tissues, potentially contributing to the hypoglycaemia associated with TNF-stimulation of immune cells during malaria infection (Hirako et al 2018). Additionally, rodent models and humans with obesity and type 2 diabetes like pathologies have elevated levels of isoleucine and dampened glucose rhythms in the blood (Lynch and Adams 2014, Isherwood et al 2017). Thus, if glucose limitation or elevation interferes with the parasite's ability to acquire time-of-day information from isoleucine, the IDC schedule will be disrupted as observed in diabetic mice (Hirako et al 2018). Connections between isoleucine and glucose might explain why the parasite protein kinase 'KIN' is involved in nutrient sensing (Mancio-Silva et al 2017).

Given that daily variation in the concentration of isoleucine in the blood of infected mice appears modest (55 μM to 80 μM from nadir to peak; Fig 5), we suggest that like *P. falciparum, P. chabaudi* is very sensitive to changes in isoleucine levels. This observation could be interpreted in 2 non-mutually exclusive ways. Perhaps this seemingly damp rhythm means that sufficient isoleucine is available around-the-clock to meet the parasites resource-needs (from the blood and scavenging of <3% isoleucine in rodent haemoglobin; Supp Fig. 1), so isoleucine acts on the IDC schedule only via a time-cue rather than as a developmental-rate limiting resource. Or, perhaps the isoleucine rhythm becomes exacerbated as infections develop high parasite burdens and hosts become sick, and so, by aligning their IDC schedule correctly early in infections, parasites minimise the costs of resource limitation later on. Differentiating between whether isoleucine only acts on the IDC schedule as a cue for an adaptive phenotypically plasticity trait and/or as a rate limiting resource would be facilitated by knowledge of the pathways involved in environmental sensing and decision making. SR10 is a likely candidate for modulating IDC duration in response to perturbations of host time-of-day (Subudhi et al 2020) and a parasite stage early in the IDC is likely responsible for time-keeping (McLean and Jacobs-Lorena 2020). Identifying KIN (Mancio-Silva et al 2017), regulated pathways and those associated with other genes recently suggested to impact the IDC schedule, including SR10 (Subhudi et al 2020), and PK7/MORC (Singh et al 2021), and determining whether they are sensitive to isoleucine might reveal how the parasites' time-keeping mechanism operates. It is also possible that parasites keep time with an endogenous clock (Reece et al 2017, Prior et al 2020). The hallmarks of an endogenous oscillator are (i) temperature compensation, (ii) free running in constant conditions, and (iii) entrainment to a Zeitgeber (Pittendrigh 1960). Recent observations are consistent with (ii) (Subudhi et al 2020, Rijo-Ferreira et al 2020, Smith et al 2020) and our results now allow entrainment to isoleucine to be tested for (iii), as well as free-running in isoleucine-constant conditions (ii), to determine whether malaria parasites possess a clock or use a simpler, reactionary strategy.

Most studies of isoleucine uptake and use in malaria parasites focus on *P. falciparum*. This parasite uses several channels and receptors (both parasite- and host-derived) to acquire resources from the host. Uninfected human RBC take up both isoleucine and methionine via the saturable L-system (Cobbold et al 2011), which supplies 20% of the necessary isoleucine (Martin and Kirk 2007). When parasitised, there is a 5-fold increase in isoleucine entering RBC which is attributable to new permeability pathways (NPPs) introduced into the RBC membrane by the parasite (Martin and Kirk 2007, McLean and Jacobs-Lorena 2020). NPPs supply 80% of the necessary isoleucine (Martin and Kirk 2007) and are active only in the host membrane at the trophozoite and schizont stages of the IDC, so suggesting this influx of isoleucine occurs only at certain times of day (e.g. after host feeding) (Kutner et al 1985). Assuming *P. chabaudi* has analogous mechanisms, we propose that elevated isoleucine is used by the parasite as a marker for a sufficiently nutrient-rich environment to traverse cell cycle checkpoints and complete the IDC (McLean and Jacobs-Lorena 2020, O'Neill et al 2020). In addition to isoleucine itself being essential, its appearance may signal a window to additional factors such vitamins, cofactors, purines, folic acid, pantothenic acid, and glucose that are required for successful replication (Sherman 1979, Müller and Kappes 2007, Müller et al 2010). In particular, that the expression of genes associated with translation are the most commonly disrupted when *P. chabaudi's* rhythms are perturbed away from the host's rhythms (Subhudi et al 2020), suggests parasites align the IDC schedule with the resources required for proteins.

Scheduling development according to the availability of the resources needed to produce progeny intuitively seems like a good strategy to maximise fitness. Yet, the costs/benefits of the IDC schedule demonstrated by parasites may be mediated by parasite density. At low parasite densities (e.g. at the start of infection), resources may be sufficient to support IDC completion of all parasites at any time-of-day, but at intermediate densities, parasites may need to align their IDC needs with rhythms in resource availability. Finally, at very high densities and/or when hosts become sick, resources could be limiting so a synchronous IDC leads to deleterious competition between related parasites. Quantifying whether the costs and benefits of a synchronous IDC vary during infections in line with parasite density and resource availability is complicated by the connection between the IDC schedule and the timing of transmission stages. By aligning the IDC schedule with the feeding-fasting rhythm which is set by light-dark rhythms in the environment, parasites benefit from the knock-on alignment of transmission stage maturation with vector biting activity (O'Donnell et al 2011, Pigeault et al 2018, Schneider et al 2018). Therefore, a key question is to what extent does the IDC schedule contribute to parasite fitness via transmission benefits versus the potential for the benefits and costs of aligning asexual replication with rhythmic resources in the blood to vary during infections? More broadly, by understanding how a single metabolite appearing in the blood of the host each day via rhythms in feeding is able to explain the timing of parasite replication, it may be possible to improve antimalarial treatments. For example, impairing the parasites ability to detect or respond to isoleucine may stall the IDC, reducing virulence and buy time for host immune responses to clear the infection, as well as preventing ring stage dormancy from providing tolerance to antimalarials (Codd et al 20011).

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Declaration of Interests
The authors declare no conflicts of interest.

Methods

Experimental designs and terminology. The same four perturbations of host and parasite rhythms were used in the metabolomics experiment and the glucose monitoring experiment. The Per1/2-null mice (non-functional proteins Period 1 & 2, backcrossed onto a C57BL/6 background for over 10 generations) were donated by Michael Hastings (MRC Laboratory of Molecular Biology, Cambridge, UK) and generated by David Weaver (UMass Medical School, Massachusetts, USA). Wild type C57BL/6 mice were housed in a 12h light: 12h dark regime (12 hours of light followed by 12 hours of darkness) and the Per1/2-null mice housed in constant darkness (DD) for 2 weeks prior to the start of infections and throughout sampling. We refer to time-of-day using ZT (Zeitgeber Time) for mice housed under entrained conditions (light:dark cycles) and hours when Per1/2-null mice are housed under constant conditions (dark:dark). WT mice either had access to food at night (dark feeding, DF) or in the day (light feeding, LF). Per1/2-null mice either had access to food for 12 hours (time restricted feeding, TRF) or constant access to food (ad libitum feeding, ALF). Every 12 hours, food was added/removed accordingly from the DF, LF and TRF cages and the cages were checked for evidence of hoarding, which was never observed. ALF cages were also disturbed during food removal/ provision of the other groups.

Sampling. All mice were infected intravenously with *P. chabaudi* DK genotype by intravenous injection of with 1×10^5 infected RBC at ring stage. Sampling started on day 5 post infection and occurred every 2 hours for both the metabolomics and glucose experiments. We made a thin blood smear each time a mouse was sampled to quantify parasite rhythms by counting ~100 parasites per blood smear using microscopy. Following (Prior et al 2018, O'Donnell et al 2019 and Rijo-Ferreira et al 2020), we used the proportion of ring stages as a phase marker (an estimate of the timing of parasite development in the blood) of parasite rhythms. We calculated amplitude and time-of-day of peak for each treatment group using sine and cosine curves in a linear model to confirm the IDC schedules for each group as used in O'Donnell et al (2019).

Metabolomics experiment. We infected 68 eight-week-old female mice: 35 C57BL/6 wild type animals (DF and LF mice) and 33 Per1/2-null TTFL clock-disrupted mice (TRF and ALF) (Supp Table 2). We sampled mice in blocks (A-D), meaning each individual mouse was sampled every 8 hours during the 26-hour sampling window, with 14 time points in total. We did not sample each mouse at each sampling point to minimise the total volume of blood being taken. At each sampling point for each designated host, 20 µl blood was taken from the tail vein to provide 10 µl blood plasma for snap freezing using dry ice.

Glucose experiment. We infected 20 eight-week-old male mice: 10 C57BL/6 wild type animals and 10 Per1/2-null circadian clock-disrupted mice (as described above, Supp Table 2). We recorded blood glucose concentration from all mice every 2 hours by taking 1 µl blood using an Accu-Chek Performa Nano glucometer (https://www.accu-chek.co.uk/blood-glucose-meters/performa-nano).

Targeted metabolomics. We quantified metabolites by analysing plasma samples using the AbsoluteIDQ p180 targeted metabolomics kit (Biocrates Life Sciences AG, Innsbruck, Austria) and a Waters Xevo TQ-S mass spectrometer coupled to an Acquity UPLC system (Waters Corporation, Milford, MA, USA) (Isherwood et al 2017, Skene et al 2018). We prepared the plasma samples (10 µl) according to the manufacturer's instructions, adding several stable isotope–labelled standards to the samples prior to the derivatization and extraction steps. Using UPLC/MS (ultra performance liquid chromatography/mass spectrometry), we quantified 185 metabolites from 5 different compound classes (acylcarnitines, amino acids, biogenic amines, glycerophospholipids, and sphingolipids). We ran the samples on two 96-well plates, randomised the sample order and ran three levels of quality control (QC) on each plate. We normalised the data between the plates using the results of quality control level 2 (QC2) repeats across the plate (n=4) and between plates (n=2) using Biocrates METIDQ software (QC2 correction). Metabolites were excluded if the CV% of QC2 was > 30% or if all 4 groups contained > 25% of samples that were below the limit of detection, below the lower limit of quantification, or above the limit of quantification or blank out of range. The remaining 134 quantified metabolites comprised of 7 acylcarnitines, 19 amino acids, 15 biogenic amines, 79 glycerophospholipids and 14 sphingolipids (see Supp Fig 3).

Isoleucine response experiments. To test the effect of the amino acid isoleucine on the IDC we compared parasite developmental progression in cultures with and without isoleucine (50 mg/L), as well as after different durations (7, 14, 18 hours) of isoleucine starvation. We set up N = 112 cultures (from 8 mice) so that for each time point within each treatment, an independent culture was sampled, avoiding any bias associated with repeat-sampling individual cultures. We used eight-week-old wild type female mice, MF1 strain, housed in a 12h:12h light:dark regime before and during infection. We infected mice intraperitoneally with 1×10^6 *P. chabaudi* genotype DK infected red blood cells at ring stage and terminally bled them on day 6 post infection, when the parasitaemia was around 15%. Mice were bled at ZT4, when parasites were late rings/ early trophozoites (see Prior et al 2018). Approximately 1 ml of blood was collected from each mouse which was then split equally across cultures in all 5 treatment groups.

Culturing. We washed infected blood twice with buffered RPMI containing no amino acids (following Spence et al 2011, see Supp Mat "Parasite culture protocol" for more details), before being reconstituted in the RPMI medium corresponding to each treatment (containing isoleucine, or not). We cultured parasites in 96-well round bottom plates with total culture volumes of 200-250 µl, at ~3% haematocrit and kept the culture plates inside a gas chamber which was gassed upon closing with 88% nitrogen 7% carbon dioxide and 5% oxygen, and then placed inside a 37°C incubator. The culture medium was custom made RPMI from Cell Culture Technologies, Switzerland (http://www.cellculture.com) (see Supp Mat "Parasite culture protocol").

Sampling. We sampled parasites in the first experiment (comparing IDC completion in isoleucine rich versus isoleucine free media) at 13-14, 16-18, 20-21, 24-26 and 27 hours after culture initiation. We also sampled parasites that had been isoleucine deprived for 7, 14 or 18 hours at 6 and 9 hrs after isoleucine addition. Samples consisted of a thin blood smear from each culture fixed with methanol and Giemsa stained. We measured the proportion of parasites in the schizont stage (as an indicator of parasites having completed their IDC) by counting ~300 parasites per blood smear.

Statistical analysis. We defined daily rhythmicity in the concentrations of the 134 metabolites detected by the UPLC/MS-MS platform as the detection of rhythmicity in at least 2 of the following circadian analysis programmes: ECHO (https://github.com/delosh653/ECHO), CircWave (https://www.euclock.org/results/item/circ-wave.html) and JTK_Cycle (https://openwetware.org/wiki/HughesLab:JTK_Cycle). For metabolites not found to be rhythmic by at least 2 of the circadian analysis programmes, we also carried out Analysis of Variance to identify metabolites that varied across the day but without a detectable 24 h rhythm (Supp Table 3 for breakdown of which candidate metabolites were rhythmic in each programme). To calculate the acrophase (timing of peak) we used linear mixed-effects regression models containing sine and cosine terms on all metabolites that varied across the day. Metabolites whose acrophase fell in the same 12h feeding or fasting window (ZT0-12 or ZT12-24) in LF and TRF infections but fell in the opposite window for the DF group were shortlisted as potential candidates to connect host feeding rhythms and the IDC schedule. We compared schizont proportion and the density of combined IDC stages using linear regression models. To avoid overfitting due to small sample sizes, "Akaike information criterion-corrected" (AICc) values were calculated for each model, and a change in 2 AICc (ΔAICc = 2) was chosen to select the most parsimonious model (Bewer et al 2016).

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Figures

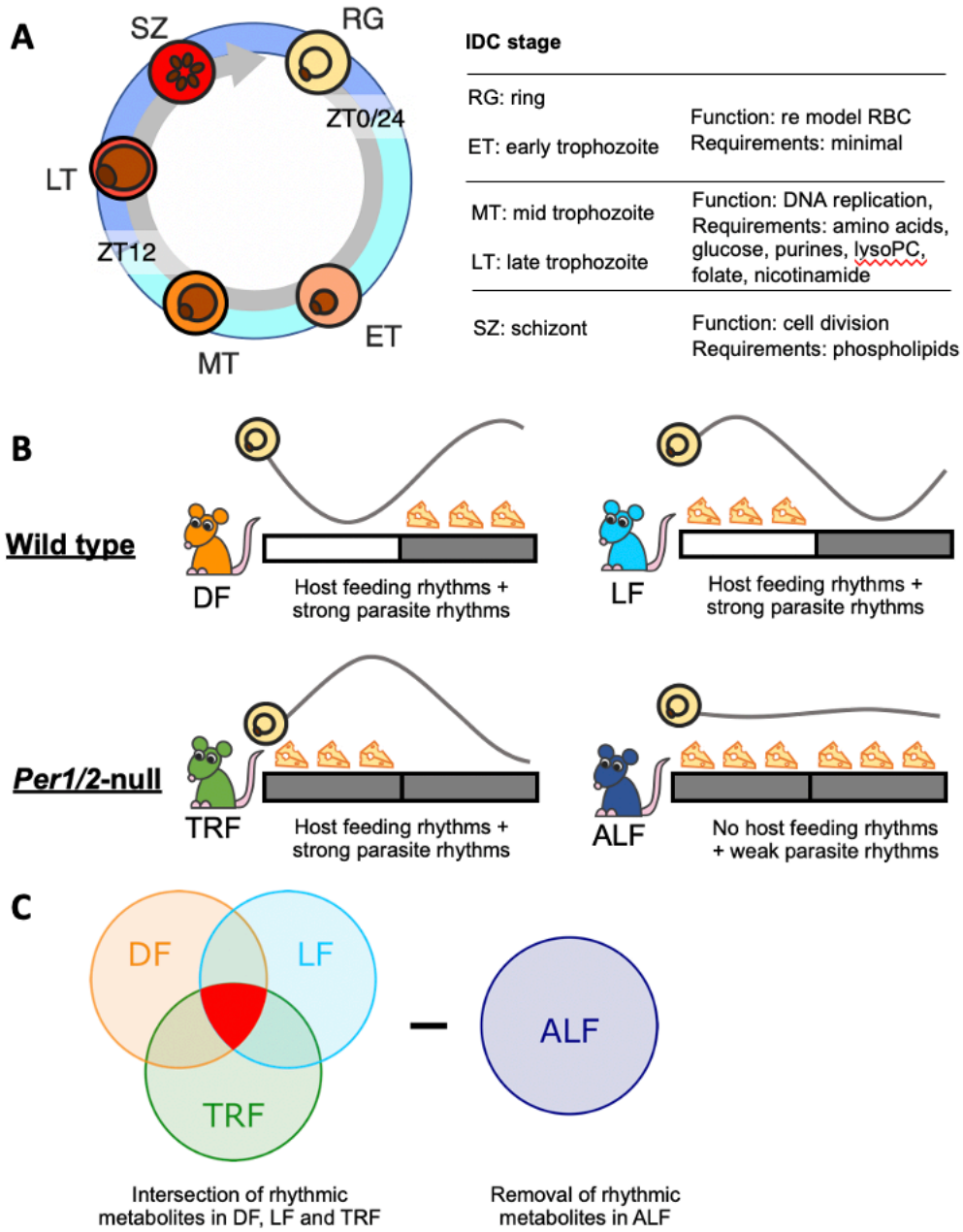

Fig. 1| Rhythms in the intraerythrocytic development cycle and host rhythms. a, The role of each IDC stage during cycles of asexual replication and resources known to be essential. RG=ring stage, ET=early stage trophozoite, MT=mid stage trophozoite, LT=late stage trophozoite, SZ=schizont. **b**, Experimental design housed wild type mice were in a 12h light: 12h dark regime (indicated by the light-dark bar) with unrestricted access to food for 12 hours either during the night-times (DF, dark feeding) or the day times (LF, light feeding) as indicated by the position of the cheeses. Per1/2-null mice were housed in continuous darkness (DD) and either experienced cycles of 12-hours with food followed by 12 h without access to food (TRF, time restricted feeding,) or given constant access to food (ALF, *ad-libitum* feeding). The parasite IDC is rhythmic in DF, LF and TRF mice but not ALF mice (O'Donnell et al 2019), and so, the IDC rhythm is substantially dampened. **c,** Metabolites that were significantly rhythmic in DF, LF and TRF mice (highlighted in red), but not in ALF mice, were sought. Treatment groups colour coded throughout: DF=orange, LF=light blue, TRF=green, ALF=dark blue.

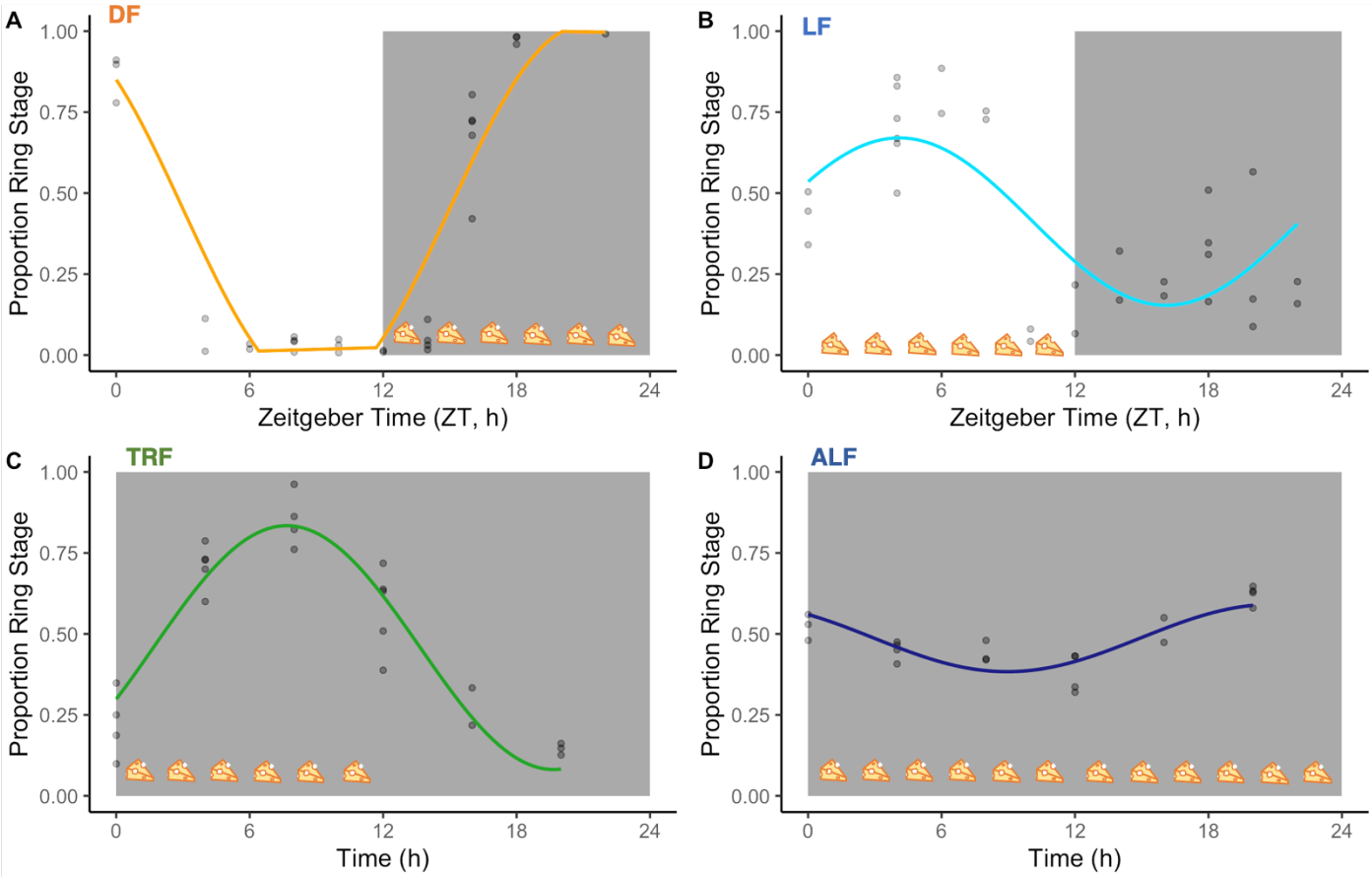

Fig 2. | IDC schedule and host feeding rhythms. The proportion of parasites at ring stage as a phase marker, for: **a,** DF: dark feeding WT mice food access ZT 12-24 (12 h at night) in 12h:12h light:dark, **b,** LF: light feeding WT mice food access ZT 0-12 (12 h in day) in 12 h:12 h light:dark. **c,** TRF: time restricted feeding Per1/2-null mice food access 0-12 hours (12 h at the same time (GMT) as LF mice) in constant darkness (DD). **d,** ALF: ad libitum feeding Per1/2-null mice access in constant darkness (see Fig 1 for Experimental Design and more details). Feeding-fasting rhythms are indicated by cheeses, the white panel denotes lights on (Zeitgeber Time=0-12 h), dark grey panel denotes lights off (Zeitgeber Time=12-24 h for DF and LF, 0-24 h for TRF and ALF). Model fits for each group are plotted on the raw data (n= 2-5 infections per time point). The fitted sine/cosine curve for DF **a** is distorted due to a large amplitude which exceeds the bounds possible for proportion data (between 0 and 1) so is truncated for visualisation. The patterns for groups are explained better by sine/cosine curves than by a straight line (see Supp Table 1),

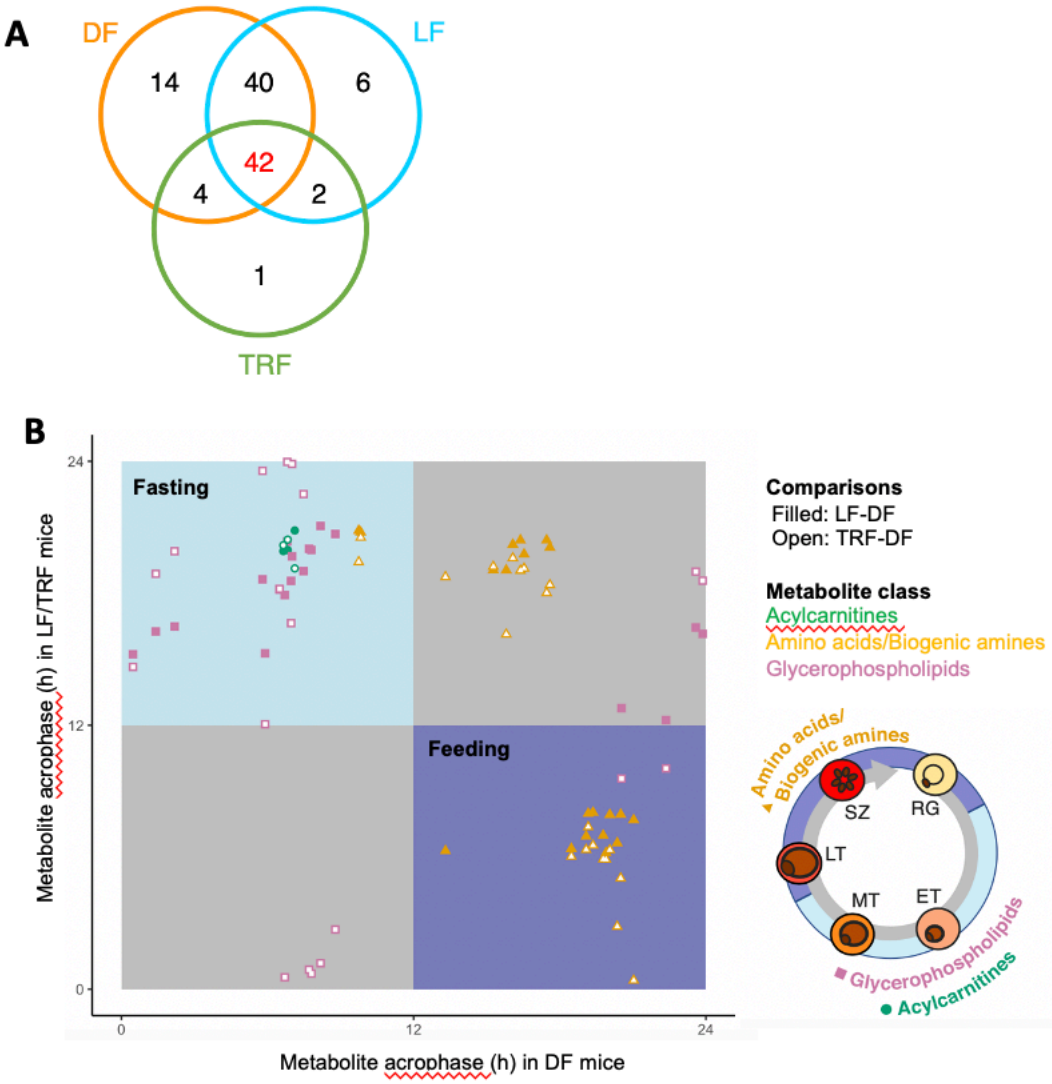

Fig 3. | Rhythmic metabolites that associate with IDC timing and host feeding-fasting cycles. a, The numbers of rhythmic intersecting metabolites out of a total of 109. **b,** Peak timing (phase) concentration of the 42 metabolites that are rhythmic in DF, LF and TRF infections. Top left panel (light blue) denotes peaks during the fasting phase and the bottom right panel (purple) denotes peaks in the feeding phase, yielding 33 candidates linked to the feeding-fasting cycle. Metabolite classes: acylcarnitines=green circles, amino acids/biogenic amines=orange triangles, glycerophospholipids=purple squares. See Supp Table 5 for peak times of each metabolite. Ring stages (RG), late trophozoites (LT) and schizonts (SZ) peak during the hosts feeding period (purple) when some amino acids/biogenic amines peak. Early trophozoites (ET) and mid trophozoites (MT) peak during host fasting (light blue) when glycerophospholipids and acylcarnitines peak.

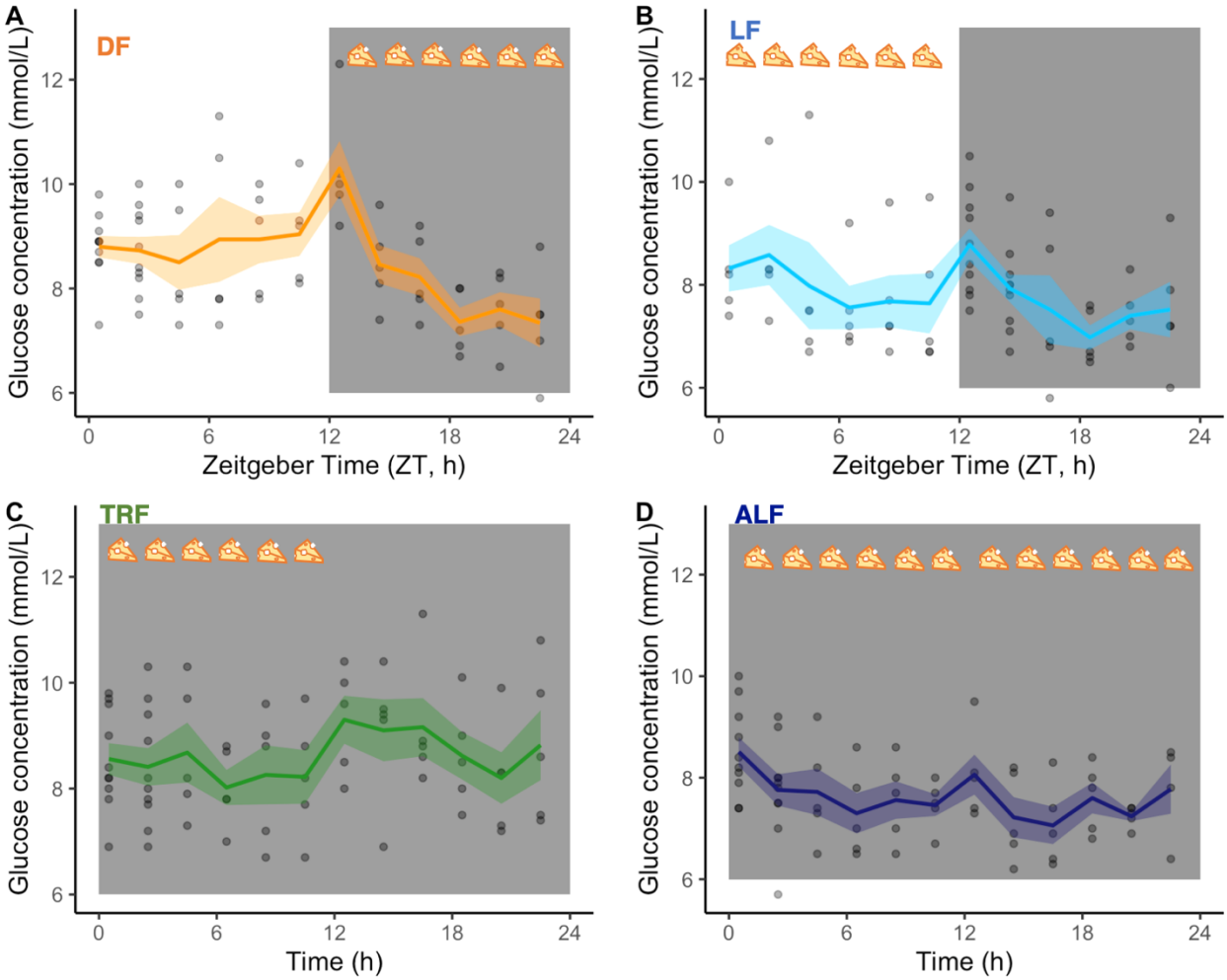

Fig 4. | Blood glucose concentrations (mmol/L). a, DF: dark feeding WT mice food access ZT 12-24 (12 h at night) in 12h:12h light:dark, **b,** LF: light feeding WT mice food access ZT 0-12 (12 h in day) in 12 h:12 h light:dark. **c,** TRF: time restricted feeding Per1/2-null mice food access 0-12 hours (12 h at the same time (GMT) as LF mice) in constant darkness (DD). **d,** ALF: ad libitum feeding Per1/2-null mice access in constant darkness (see Fig 1 for Experimental Design and more details). Feeding-fasting rhythms are indicated by cheeses, the white panel denotes lights on (Zeitgeber Time=0-12 h), dark grey panel denotes lights off (Zeitgeber Time=12-24 h for DF and LF, 0-24 h for TRF and ALF). The lines and shading are mean ± SEM at each time point and the dots are the raw data. At time points 0.5 and 2.5 for DF, TRF and ALF, and 12.5 and 14.5 for LF the points are stacked because the time course lasted ~26 hours and the data are plotted on a 0-24 hour axis.

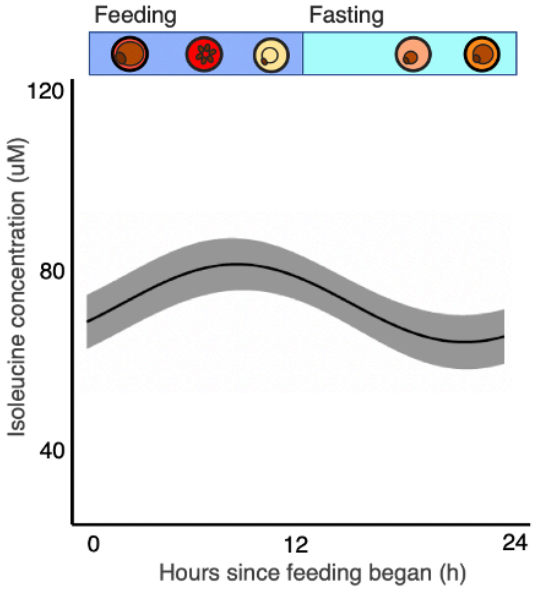

Fig 5| Isoleucine is rhythmic and coincides with the IDC schedule and host feeding-fasting cycles. Model fit (best fit line and 95% prediction interval) for DF, LF and TRF infections combined, from the time since feeding commences, with parasite IDC stages and feeding-fasting windows overlaid. Stage peaks and intervals taken from Prior et al (2018) and Fig 2.

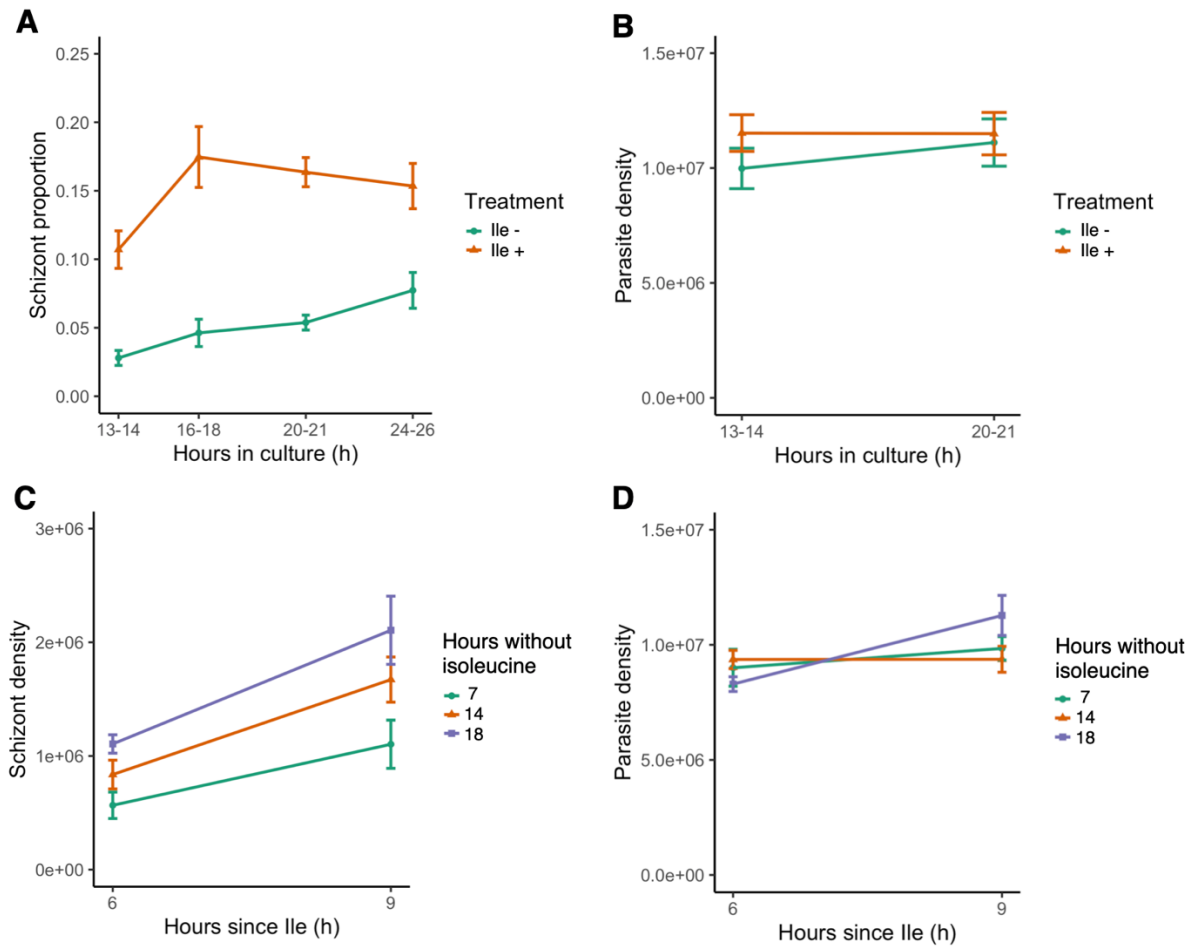

Fig 6. | Isoleucine provision and withdrawal drives IDC completion but does affect parasite mortality. a, IDC completion defined as the proportion of parasites that are schizonts, in cultures with (orange triangles, Ile +, 50 mg/L) or without (green circles, Ile -) isoleucine. **b,** Density of all parasite stages when parasites are cultured with or without isoleucine. Density of **c,** schizonts and **d,** all parasite stages, after the addition of isoleucine into cultures following isoleucine deprivation for 7 (green circles), 14 (orange triangles) and 18 hours (purple squares). The proportion of schizonts in the blood seeding the cultures was ~0.005.

Tables

Metabolite class & general function	Role in the IDC?	Putative time-cue?
Acylcarnitines • Energy metabolism • Mitochondrial function • Fatty acid transport	No evidence a lack of acylcarnitines affects either the IDC schedule or replication rate. Peak during fasting when parasites are in their least energetically/ metabolically demanding stages.	Very unlikely.
Glycerophospholipids • Cellular signalling and trafficking • Membrane neogenesis • Haemozoin formation	Phospholipid composition of the host RBC increases during infection, especially oleic acid (18:1) (Déchamps et al 2010). Parasites scavenge fatty acids and also lysoPCs from host plasma, which compete with each other as a source of the acyl components required for lipid synthesis. Lysophosphatidylcholine a C18:1 (lysoPC a 18:1), which contains an oleic acid side chain, is associated with IDC progression. Majority peak during host fasting when parasites are in their least energetically/ metabolically demanding stages. Thus, to be a time-of day cue	Unlikely. Not essential; unlike the liver stage, IDC stages can synthesise fatty acids de novo via type II FA synthase (FASII), (Tarun et al 2009).
Biogenic amines and amino acids: • Nucleic acid and protein synthesis	Parasites must scavenge several amino acids from the host, including six host-'essential' (isoleucine, leucine, methionine, phenylalanine, threonine and valine) and one host-'non-essential' (alanine) amino acid (Payne and Loomis 2006) that are rhythmic. Parasites can scavenge most amino acids from catabolism of host haemoglobin (Liu et al 2006, Babbitt et al 2012, Martin and Kirk 2007). Isoleucine is the only amino acid absent from human haemoglobin and is one of the least abundant amino acids in rodent haemoglobin (1-3%, Supp Fig 1) yet makes up 9% of both *P. falciparum's* and *P. chabaudi's* amino acids (Yadav and Swati 2012). The response of *P. falciparum* to isoleucine withdrawal is not influenced by whether they were previously cultured in a high or low isoleucine concentration environment and results in dormancy that can be broken upon isoleucine replenishment (Babbitt et al 2012). Coinciding with the rise in isoleucine concentration during the feeding window, trophozoites transition to schizonts before bursting and beginning development as ring stages at the end of the feeding window (Fig 5).	Likely. Isoleucine is the only essential amino acid for *P. falciparum*, it cannot be stored, and dormancy has no apparent ill-effects on subsequent development. *P. falciparum* deprived of other amino acids develop as normal (Lui et al 2006)

Table 1| Potential for acylcarnitines, glycerophospholipids and amino acids/amines candidates that associate with the feeding-fasting cycle to explain the IDC schedule. Requirements for these metabolites is likely to increase as each parasite cell progresses through its IDC.