The evolutionary ecology of biological rhythms in malaria parasites

Aidan J. O'Donnell

Thesis submitted for the degree of Doctor of Philosophy

THE UNIVERSITY *of* EDINBURGH

School of Biological Sciences

2021

Lay Summary

Biological rhythms with durations of around 24 hours are thought to have evolved to allow organisms to organize their activities with the environmental rhythms resulting from the rotation of the Earth. The genetic and molecular mechanisms controlling biological rhythms are well understood but it is still unclear why they have evolved and what benefits they provide, especially for parasites that live inside the bodies of other organisms. Daily rhythms in parasites are wide ranging and include rhythms in transmission to new hosts, rhythms in growth and metabolism and rhythms in defenses that are scheduled to match the rhythmic immune responses of the host. Malaria parasites (*Plasmodium*) exhibit daily rhythmicity in their cycle of development within red blood cells of their host and the time-of-day that specific developmental stages occur is coordinated with the time-of-day that their hosts feed. For malaria species that develop synchronously every parasite in the infection undergoes their development at the same schedule (i.e. each parasite is at the same stage at the same time-of-day), but asynchronous malaria species also exist in which each parasite in the infection is at a different schedule to each other. I use a rodent malaria model system (primarily with a synchronous species, *P. chabaudi*) to explore the coordination of parasites with their hosts and understand what benefits, if any, host-parasite synchrony provides to the parasite.

I demonstrate that aligning the parasite development cycle with the host's feeding patterns is beneficial to parasite growth, particularly early (within 48h) in the infection, but find that this benefit is specific to infections started with a specific parasite developmental stage. I reveal that the parasite's coordination with the host is independent of the host's biological clock genes and is primarily driven by the host's feeding rhythms. I also show that when parasites start out of alignment with host feeding rhythms (analogous to jet lag) they reschedule by shortening the duration of their development, which moves their development cycle earlier by ~2 hours each day until they are fully aligned with the host. I find that an asynchronous malaria parasite species, *P. berghei*, is resistant to the conditions that lead to host-parasite synchrony in *P. chabaudi*, suggesting there are specific ecological reasons for this parasite to develop asynchronously. Finally, I reveal that changes to the blood feeding rhythms of the Asian malaria mosquito (*Anopheles stephensi*), the vector for malaria parasites, result in small changes to the timing of its reproduction but that, overall, such changes will not impact malaria transmission.

These findings are important because understanding how parasites 'keep time' may provide new targets for interventions such as drugs that disrupt the parasite's development schedule. More generally, our results provide a demonstration of the value of biological rhythms to an organism and the role that rhythms have in shaping the outcomes of a globally important infectious disease.

Abstract

Biological rhythms are a ubiquitous feature of life and are assumed to allow organisms coordinate their activities with daily rhythms in the abiotic environment resulting from the rotation of the Earth every 24 hours. The genes and molecular mechanisms underpinning circadian clocks in multicellular organisms are relatively well understood in contrast to the evolution and ecology of circadian rhythms. Circadian rhythms mediate interactions between organisms; from predators and prey, to mating behaviours between males and females, to hosts and parasites. The role of daily rhythms in infections is gaining traction because explaining the regulatory mechanisms and fitness consequences of biological rhythms exhibited by parasites and hosts offers new avenues to treat infections. Here, I explore how periodicity in parasite traits is generated and why daily rhythms matter for parasite fitness. My work focuses on malaria (*Plasmodium*) parasites which exhibit developmental rhythms during replication in the mammalian host's blood and during transmission to insect vectors. Rhythmic in-host parasite replication is responsible for eliciting inflammatory responses, severe anaemia, 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.

My papers integrate an evolutionary ecology approach with chronobiology and parasitology to investigate how host-parasite-vector interactions shape the evolution of rhythmicity in parasites traits. I have used a rodent malaria parasite model system (*Plasmodium chabaudi*) for my experiments, capitalising on the tractability of this model for the human malaria, *P. falciparum*. *P. chabaudi* exhibits a 24-hour rhythm in replication, facilitates ecologically realistic studies because experiments can be carried out *in vivo* (compared to the *in vitro* limitations on studying human parasites), and perturbations to the timing of the in-host and in-vector environments are straightforward. My findings include:

1) Perturbing the timing of parasite rhythms with respect to the timing of host rhythms (analogous to giving the parasites "jet lag"), results in a fitness cost to the parasites, evident by a 50% reduction in both asexually replicating and transmission stage parasites.
2) The consequences of temporal mismatch to the host manifest very early in the infection (within 48 hours, i.e. the first 1-2 cycles of replication) and are dependent on the parasite stage by which infections are initiated (0-12 hour old parasites suffer a cost, whereas 12-24 hour parasites benefit).
3) The timing of the parasite replication cycle is independent of the canonical 'core' host clock (i.e. transcription translation feedback loop) and instead depends on the timing of feeding-fasting rhythms of the host.
4) If perturbed, the timing of the parasite's rhythm reschedules to regain synchrony with the timing of the host's rhythm within 7 replication cycles. Specifically, parasites achieve this by speeding up the replication rhythm by 2-3 hours per cycle, and the rate of rescheduling is independent of parasite density.
5) Naturally asynchronous *Plasmodium* species are 'resistant' to conditions that lead to alignment with host rhythms in synchronously replicating species. This suggests that unknown ecological differences between these parasite species selects for vastly different schedules of within-host replication rather than some species being constrained to replicate asynchronously.
6) In addition to the timing of parasite rhythms impacting directly upon within-host dynamics, timing also matters – albeit indirectly - for transmission, via impacts on the population dynamics

of the vector. For example, receiving a blood meal in the morning makes mosquitoes more likely to lay eggs, lay slightly sooner and have a larger clutch size than those feeding at night. Yet, whilst mosquitoes infected with malaria die sooner, the effects of taking a blood meal at different times of day do not impact transmission of an asynchronously replicating malaria parasite.

It is beneficial for parasites to be in synchronization with their host's feeding-fasting rhythms and plasticity in the IDC duration facilitates this synchrony by enabling parasites to make small daily changes to their IDC schedule when necessary. Understanding the extent of, and limits on, plasticity in the IDC schedule is important as it may reveal targets for novel interventions, such as drugs to disrupt IDC regulation and preventing IDC dormancy conferring tolerance to existing drugs. More generally, our results provide a demonstration of the adaptive value of biological rhythms and the utility of using an evolutionary framework to understand parasite traits.

Table of contents

Lay Summary i
Abstract ii
Table of contents iv
List of published works included in the portfolio vi
Declaration vii
Acknowledgements ix

Introduction 1
Biological timing 1
Rhythms affect fitness 1
Rhythms in infections 2
Malaria parasite rhythms 5
How are the rhythms of malaria parasites controlled? 7
Why are malaria parasites rhythmic? 8
Why care about malaria parasite rhythms? 11
Aims & objectives 13
Methodology 14
Parasites 14
Hosts 16
Vector 17
Experimental designs 17
Quantifying host and parasite rhythms 19
Results 22
What? 22
How? 23
Phase (Timing) 23
Synchrony (Amplitude) 24
Period (IDC duration) 25
Why? 25
Within-host survival 25

Between-host transmission 26
Conclusions 27
What are the characteristics of the IDC rhythm? 27
How is the IDC rhythm established? 28
Why have parasites evolved to schedule the IDC? 29
From mice to humans 31
References 32
Appendix 1 41
Paper #1 (2021): Fitness costs of disrupting circadian rhythms in malaria parasites 44
Paper #2 (2013 & 2014): Disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated 53
Paper #3 (2019): Time-of-day of blood-feeding: effects on mosquito life history and malaria transmission 67
Paper #4 (2020): Host circadian clocks do not set the schedule for the within-host replication of malaria parasites 83
Paper #5 (2021a): Ecology of asynchronous asexual replication: the intraerythrocytic development cycle of *Plasmodium berghei* is resistant to host rhythms 92
Paper #6 (2021b): Mistimed malaria parasites re-synchronize with host feeding-fasting rhythms by shortening the duration of intra-erythrocytic development 104

List of published works included in the portfolio

The following publications are submitted as a unified portfolio investigating the biological rhythms of malaria parasites and their vectors. Each of the publications are included at the end of this thesis.

1. O'Donnell A. J., Schneider P., McWatters H. G. and Reece S. E. (2011). "Fitness costs of disrupting circadian rhythms in malaria parasites." Proceedings of the Royal Society B: Biological Sciences 278: 2429 - 2436.

2. O'Donnell, A. J., Mideo N. and Reece S. E. (2013). "Disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated." Malaria Journal 12: 372.
   And
   O'Donnell, A. J., Mideo N. and Reece S. E. (2014). "Correction: disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated." Malaria Journal 13(1): 503.

3. O'Donnell, A. J., Rund S. S. C. and Reece S. E. (2019). "Time-of-day of blood-feeding: effects on mosquito life history and malaria transmission." Parasites & vectors 12(1): 1-16.

4. O'Donnell, A. J., Prior K. F. and Reece S. E. (2020). "Host circadian clocks do not set the schedule for the within-host replication of malaria parasites." Proceedings of the Royal Society B: Biological Sciences 287(1932): 20200347.

5. O'Donnell, A. J. and Reece S. E. (2021a). "Ecology of asynchronous asexual replication: the intraerythrocytic development cycle of *Plasmodium berghei* is resistant to host rhythms." Malaria Journal 20(1): 1-12.

6. O'Donnell, A. J., Greischar M.A. & Reece S. E. (2021b). "Mistimed malaria parasites re-synchronise with host feeding-fasting rhythms by shortening the duration of intra-erythrocytic development." Parasite Immunology e12898

Declaration

I confirm that I composed this portfolio of works in which I integrate the disciplines of evolutionary ecology, chronobiology and parasitology. These published works have required collaborations to support the publishing process namely with my PI, Sarah Reece, and others (listed as authors on each publications) as follows:

Paper #1:

The experiment was designed by Sarah Reece and I. I carried out the experiment and along with Sarah Reece, I analysed the data. I wrote the first draft of the paper with Sarah Reece and Petra Schneider and Harriet McWatters contributed to interpretation of results and the writing of the manuscript.

Paper #2:

The experiment was designed by Sarah Reece and I. I carried out the experiment and Sarah Reece and myself analysed the data. I wrote the first draft of the paper with Sarah Reece and Nicole Mideo contributed to interpretation of results and the writing of the manuscript.

Paper #3:

I designed and carried out the experiment and analysed the data. I wrote the first draft of the paper with Sarah Reece and Sam Rund contributed to interpretation of results and the writing of the manuscript.

Paper #4:

I designed and carried out the experiment and analysed the data. I wrote the first draft of the paper, and Sarah Reece and Kimberly Prior contributed to interpretation of results and the writing of the manuscript.

Paper #5:

I designed and carried out the experiment and analysed the data. I wrote the first draft of the paper with Sarah Reece and Sarah Reece and I contributed to interpretation of results and the writing of the manuscript.

Paper #6:

I designed and carried out the experiment and analysed the data. I wrote the first draft of the paper, and Sarah Reece and Megan Greischar contributed to interpretation of results and the writing of the manuscript.

During the period my first author papers are selected from for this thesis, I have also contributed to a number of other papers. The full references accompanied by a brief description of the core findings can be found in Appendix 1:

Contributed considerably to designing and carrying out experimental work, interpretation of results and writing of the manuscript:

1. Subudhi A. K., **O'Donnell A. J.** *et al* (2020), Nature Communications.
2. Westwood M. L., **O'Donnell A. J.** *et al* (2020), Malaria Journal.
3. Rund S. S. C., **O'Donnell A. J.** *et al* (2016), Insects.

Contributed experimental support, and interpretation and editing of the manuscript:

4. Prior K. F., Middleton B., Owolabi A. T. Y., Westwood M. L., Holland, J., **O'Donnell A. J.** *et al* (2021), Wellcome Open Research.
5. Prior K. F., **O'Donnell A. J.** *et al* (2019), Scientific Reports.
6. Schneider P., Rund S. S. C., Smith N. L., Prior K. F., **O'Donnell A. J.** *et al* (2018), Proceedings of the Royal Society B.
7. Prior K. F., van der Veen D. R., **O'Donnell A. J.** *et al* (2018), PLOS Pathogens.

Contributed experimental samples and/or editing support for the manuscript:

8. Davidson M. S., Yahiya S., Chmielewski J., **O'Donnell A. J.** *et al* (2021), medRxiv.
9. Birget P. L. G., Schneider P., **O'Donnell A. J.** *et al* (2019), Evolution, Medicine, and Public Health.
10. Westwood M. L., **O'Donnell A. J.** *et al* (2019), Nature ecology & evolution.
11. Lippens C., Guivier E., Reece S. E., **O'Donnell A. J.** *et al* (2018), Evolutionary Applications.
12. Birget P. L., Repton C., **O'Donnell A. J.** *et al* (2017), Proceedings of the Royal Society B.
13. Schneider P., Bell A. S., Sim D. G., **O'Donnell A. J.** *et al* (2012), Proceedings of the Royal Society B.

I can confirm that none of the work in this thesis has been submitted for any other degree or professional qualification.

Aidan J. O'Donnell, December 2021

Acknowledgements

Firstly, I'd like to give a huge thanks to my supervisors Sarah Reece and Petra Schneider. As my boss and my colleague they have been integral to all my research, gave me a career as a scientist and have enriched my life in Edinburgh greatly. Sarah has a skill in turning around my self-doubt and always encouraged me that the work we've completed was indeed cool and exciting. Petra always made sure I took breaks so that I wouldn't burn myself out and never got annoyed whenever I regularly needed stats or lab support. They're both genuinely nice people and have been great friends for over 13 years.

I also thank all my collaborators: Nicole Mideo, Megan Greischar, Nick Savill, Bert Maier, Daan van der Veen, and Harriet McWatters. They have been integral to interpreting results and coming up with future avenues of research. Working with scientists from different fields has made me approach research with a broader brush and I think my work is better because of it. They also give me someone to drink with at conferences, which is always a bonus.

I'd like to thank my 'lab amigos', Kim Prior and Sam Rund who were always there to hear my rants, come up with cool new experiments, try new restaurants and were awesome international travel buddies. I'm really happy we're friends.

I'd like to thank everyone in lab: Aliz, Alejandra, Mary, Jacob, Catherine, 'Honest/Jackpot' Ron and the lab alumni Laura, Ricardo, Lucy, Vincent, Philip, Charlotte and the Honours students. Without your help I would never have got all the sampling done or ever been able to learn the stats. I'm sorry for all the overnights, I really do appreciate the help.

To my partner David, you put up with my weird hours and stress during big experiments. You always look out for me and make me want to be a better person. But now, you can say your partner is a doctor so I feel like that's us even.

Finally, thank you to all the mice and mosquitoes that were part of my research. You did very well.

Introduction

Biological timing

Biological rhythms are a ubiquitous feature of life that is assumed to have evolved to allow organisms to coordinate their behaviours and physiological processes in line with the predictable consequences of the Earth's daily and seasonal rotations. Rhythms can result from reactionary responses to cyclic stimuli (such as sunlight) or may be controlled by internal clocks (oscillators) which allow organisms to anticipate the stimulus before it occurs. When a 24h biological rhythm is linked to an internal self-sustaining time-keeping oscillator (i.e. persists in constant conditions; 'free-running'), is entrainable to outside stimuli (such as light or heat), and can be maintained across varied temperatures, it is deemed to be circadian (derived from the latin *circa* meaning 'about' and *dian* meaning 'day'). A key advantage to using a circadian clock is that it allows organism to anticipate an environmental change before it happens thus maximizing their ability to prepare to exploit transient resources or to better survive upcoming challenges.

The mechanisms underpinning circadian clocks have been described for cyanobacteria (Kondo et al. 2000), plants (Johansson et al. 2019), insects (Hardin 2011) and mammals (Takahashi 2017). Many of the clocks share a similar design based on transcription-translation feedback loops (TTFLs) in which clock genes are auto-regulated by their protein products (termed a 'canonical clock'), but the components of the clock (genes/proteins) vary between species (Dunlap 1999). As some of the protein components are light-sensitive, the entire clock is kept in synchrony with the environment by changes in light/dark when day/night occur (however some clocks can also be set by feeding-fasting rhythms and temperature). Not all biological clocks rely on TTFL loops; an ancient timekeeping mechanism driven by a non-transcriptional oscillator operates in red blood cells which have no transcriptional/translational machinery and in algae that are unable to use their TTFL (O'Neill et al. 2011, Edgar et al. 2012, Feeney et al. 2016), suggesting fundamental clock mechanisms are yet to be understood.

Rhythms affect fitness

A wide range of important biological processes from gene expression, protein function, immune defenses, to daily and seasonal behaviours, are subject to regulation by the clock. For example, at least 40% of genes of mice are rhythmically expressed in at least one tissue (Zhang et al. 2014). Biological rhythms are thought to provide extrinsic adaptive value to organisms through the coordination of their behaviour and physiologies with the external environment, as well as intrinsic adaptive value by compartmentalizing incompatible internal processes to different times-of-day (Sharma 2003). However, empirical evidence of either extrinsic or intrinsic value are rare because it is difficult to modify the timing of organisms in an ecologically relevant way. Thus, over the last few decades, the field of chronobiology has focused efforts on discovering the mechanisms behind rhythms rather than their consequence for fitness.

The fitness benefits of biological rhythms have been most clearly demonstrated for cyanobacteria and *Arabidopsis* (Ouyang et al. 1998, Dodd et al. 2005, Rubin et al. 2017, Hellweger et al. 2020). Individuals raised in an environmental cycle that matches the duration (period) of the endogenous clock grow/replicate faster, are of better quality, and have a competitive advantage over individuals for which

their endogenous clock runs with a longer/shorter period than the environmental rhythm (Ouyang et al. 1998, Dodd et al. 2005). Termed the resonance hypothesis, this phenomenon has also been observed in lab reared *Drosophila* (Pittendrigh et al. 1972, Horn et al. 2019), in wild caught mosquito larvae (*Wyeomyia smithii*) (Emerson et al. 2008) and in the fungus *Neurospora discreta* (Koritala et al. 2020). Other observations that suggest clocks enhance fitness via extrinsic benefits include: Disrupting the clock of chipmunks (through lesions of organs maintaining the suprachaismatic clock or by genetic modification) increases mortality during the hibernation season (DeCoursey et al. 1998, DeCoursey et al. 2000), and a disrupted clock causes a reduction in lifespan and reproductive output of *Drosophila* (Beaver et al. 2002). In a long-term wild release study, mutant mice with a shorter (<24h) circadian period length exhibit reduced survival and reduced fecundity compared to mice with near 24 hour periods and consequently the prevalence of the 'short-clock' gene mutation in the population dropped from ~50% to 20% in only 14 months (Spoelstra et al. 2016).

The intrinsic value of rhythms are more difficult to determine. Natural selection has maintained clocks in organisms that live in constant conditions such as cave-dwelling fish and millipedes that manifest as rhythms in locomotor activity, suggesting internal rhythms are important (Koilraj et al. 2000, Cavallari et al. 2011). Further, *Drosophila* raised in constant conditions for over 600 generations do not lose circadian rhythmicity in eclosion despite the fact that selectively neutral traits in this species are usually lost within 100 generations (Sheeba et al. 1999, Sharma 2003). Intrinsic benefits of rhythms have been demonstrated in yeast in which rhythms in cell division (when constrained to the dark period) minimizes rates of genetic mutation (Chen et al. 2007), and in mice, clock genes play a critical role in circadian metabolism with knockouts of these genes resulting in a wide array of metabolic diseases (Marcheva et al. 2010, Doi 2012, Kim et al. 2020).

In addition to rhythms in the abiotic environment, and internally, rhythms of other organisms form part of the periodic environment. This includes interactions between hosts and mutualists, for example, composition of rhizosphere bacterial communities are directly influenced by the *Arabidopsis* clock and bacterial communities associated with wild type plants provide growth benefits to the host plant compared to rhizosphere communities associated with clock mutant plants (Hubbard et al. 2018). Pollination success of *Nicotiana attenuata* is determined by the coordination of rhythms in flower movement and the presence of night active (hawkmoths) or day active (hummingbirds) pollinators (Yon et al. 2017). Interactions between predators and prey are also influenced by biological rhythms, for example, guillemot fledglings exhibit time-of-day differences in nest-leaving behaviour and jump off cliffs to fledge at the time-of-day predation risk is lowest (Tinbergen et al. 1979). Circadian rhythms control the migration of planktonic copepods (the largest daily migration of biomass in the world) by allowing copepods to optimize a tradeoff between feeding and avoidance from predators (including rhythms specific to younger copepods to avoid predation by adults) (Kennedy et al. 2000, Häfker et al. 2017). The circadian clock of *Arabidopsis* provides protection against herbivores by enabling the plant to upregulate defenses in a time-of-day pattern that matches the predictable feeding behaviour of the cabbage looper caterpillar (Goodspeed et al. 2012). Rhythms can also influence interactions between hosts and parasites and this avenue of research has been gaining recognition over the last decade.

Rhythms in infections

Host-parasite interactions are widespread across all taxa and given the myriad of rhythms that parasites encounter within a host it is unsurprising that biological rhythms shape infection processes (Martinez-

Bakker et al. 2015, Reece et al. 2017, Rijo-Ferreira et al. 2017, Westwood et al. 2019, Prior et al. 2020) (Note: my use of the term parasites also encompasses pathogens). Host rhythms mediate infection severity and their susceptibility to infection and onwards transmission of parasites. *Drosophila* exhibit higher mortality when infected with *Streptococcus* or *Pseudomonas* during the day instead of night and for *Streptococcus* even higher mortality was observed in flies in which the host clock is impaired (Lee et al. 2008, Stone et al. 2012). *Chlamydia* exhibits time-of-day differences in pathogenesis when infection occurs during the hosts rest phase (Lundy et al. 2019). The host clock of *Arabidopsis* influences susceptibility to infections by *Botrytis cinerea* and *Pseudomonas syringae* with lower pathogenicity when infections occur at dawn (Bhardwaj et al. 2011, Hevia et al. 2015, Ingle et al. 2015, Larrondo et al. 2018). The Asian malaria mosquito, *Anopheles stephensi*, exhibits lower bacterial burden and mortality when infected with *E. coli* during its rest phase during the day versus its evening active phase (Murdock et al. 2013). Transmission to the host (host susceptibility) and from the host to the vector (transmission) are also under the influence of host rhythms, for instance, *Listeria* infection in mice exhibits time-of-day patterns with higher colonization when infection occurs during the host's rest vs active phase (Nguyen et al. 2013). Host circadian clocks directly influence the success of entry into cells and dissemination through tissues of several viruses, such as, Sars-coV-2 (Zhuang et al. 2021), hepatitis B (Zhuang et al. 2021), murine herpes and influenza (Edgar et al. 2016). These interactions can have negative consequences for the host, for example, wild-type cells with an intact clock are more susceptible to hepatitis C infection compared to cells in which the clock gene *Bmal1* was knocked out (Zhuang et al. 2019). Opportunities for transmission are also influenced by the rhythms of secondary hosts/vectors. For example, some trematode species are thought to coordinate the time-of-day of emergence of transmission stages from their snail host with the activity of their fish host (Faltýnková et al. 2009, Hannon et al. 2018)(parasite coordination with vectors is further discussed below). While there is a lot of variation dependent on specific host-parasite combinations, overall, a general pattern is that host processes that protect against infection are assumed more effective during the host's active phase and both mortality and severity of infections are higher when infection occurs during the rest phase (Westwood et al. 2019).

Circadian rhythms in immune responses are assumed to explain why the time-of-day of infection matters for hosts (Reece et al. 2017, Westwood et al. 2019, Prior et al. 2020). Indeed, rhythms in mammalian immune responses are well documented (Scheiermann et al. 2013, Dumbell et al. 2016, Scheiermann et al. 2018, Baxter et al. 2020). Many pro-inflammatory responses to pathogens and their byproducts are upregulated in anticipation of an organism's active phase when the organism is assumed most likely to encounter infectious agents, thus mitigating the chance of infection. For example, in humans, cytokines such as tumour necrosis factor (TNF) and interleukin-1β (IL-1β) are upregulated during the day (the human's active phase), whereas they are upregulated at night in mice (which are nocturnal) (Dimitrov et al. 2009, Keller et al. 2009, Scheiermann et al. 2013). Rhythms in the activity of immune cells also impacts upon infectivity, for example, clock driven rhythms within host monocytes regulate the magnitude of *Leishmania spp* infection with increased infection late in the day, correlating with the peak time-of-day that the infiltration of macrophages (a host cell for *Leishmania*) to the infection site occurs (Nguyen et al. 2013, Kiessling et al. 2017). Similar time-of-day specific responses occur in *Salmonella* infection of mice (Bellet et al. 2013). When infected during the rest phase mice experienced higher rates of colonization and exhibited higher pro-inflammatory responses compared to infection during the active phase.

The immune responses as a driver of rhythms in infections has received the most attention but access to resources that parasites require are also rhythmic and impact upon parasite activities. For example, the anterior-posterior diurnal migration of the rat tapeworm *Hymenolepis diminuta* along the host's gut is proposed to be an adaption to maximize resource intake by following food as it progresses through the gut (Platt et al. 2010). Gene ontology analysis of *Trypanosoma* parasites found that 10% of their genes are under clock control and while some of these genes are thought to allow the parasites to tolerate host immune responses and drug treatment (Rijo-Ferreira et al. 2017) several rhythmic genes relate to feeding, such as carbohydrate metabolism. These feeding related genes peak at a timing that corresponds with the end of the hosts feeding schedule when such resources are available in the blood (Rijo-Ferreira et al. 2017). *Daphnia* feeding rhythms influence their within-host mutualistic microbiomes. Despite two *Daphnia* species cohabitating the same freshwater environment, their bacterial microbiome composition is significantly different and is thought to be directly influenced by differences in the time-of-day each species feeds (Pfenning-Butterworth et al. 2021). However, in contrast to the expectation that host feeding rhythms may also influence the rhythmicity of *Schistosoma* parasites in mammals, transcriptomics revealed no evidence of daily rhythms in genes correlated with blood feeding, despite a 'rush hour' in the parasite's metabolic processes observed at the start of the host's rest period (Rawlinson et al. 2021). The influence that rhythms in host resources have on parasites is often overlooked and more research into the role of these rhythms in infections are needed.

In addition to infection processes being shaped by host rhythms, parasites possess their own rhythms. For example, the fungus *Botrytis cinerea* utilizes its own TTFL clock to anticipate rhythms of its host, *Arabidopsis*, increasing its expression of virulence genes at dusk when the host upregulates its defenses (Hevia et al. 2015). *Trypanosome brucei* also has a circadian clock (although the specific clock components are still unknown), which is thought to align its metabolism with its host's feeding-fasting rhythm (Rijo-Ferreira et al. 2017). *Trypanosome brucei* and *Botrytis cinerea are* the only parasites known to fulfil all criteria for having a circadian clock so it's currently not known how other parasites keep time. This may be due, in part, to the fact that identifying potential clock mechanisms across species is difficult because there is little homology in clock components across taxa.

Most research on parasite rhythms has concerned whether parasites can capitalize on daily rhythms in transmission opportunities. For example, the coccidian parasite, *Isospora turdi*, sheds it transmission forms at the time-of-day that minimizes oocyst mortality from UV exposure, thus increasing the survival of transmission stages whilst they wait to be ingested by a new host (Martinaud et al. 2009). The transmission forms of at least 10 species of *Schistosoma* exhibit time-of-day differences in emergence from their intermediate snail host when seeking a definitive host. The time-of-day for shedding depends on whether the most abundant definitive host species in the region is nocturnal or diurnal because parasites are most likely to encounter hosts in their active phase (Lu et al. 2009, Su et al. 2013). As well as aligning timing with the rhythms of the next host, the filarial nematode, *Wucheria bancrofti*, also coordinates with the biting rhythm of its mosquito vector (Hawking 1967). In the host, *Wucheria bancrofti* spends the day deep in tissues (e.g. lung) where conditions are favourable for growth but in the evening when its mosquito vector is most active, the worms migrate to the host's peripheral capillaries to maximize uptake in a blood meal (Hawking 1967). The time-of-day the worm migration occurs is specific to the timing of the local vector and can be either nocturnal (transmitted by night-biting *Anopheles* and *Culex* mosquitoes) or diurnal (transmitted by day biting *Aedes*) (*Figure 1*). Perhaps the most sophisticated strategy for exploiting rhythmic transmission opportunities is the lancet river

fluke (*Dicrocoelium dendriticum*), which manipulates the behaviour of its ant host in a time-of-day manner, causing it to move to and remain at the best location to be ingested by cattle (the parasite's intermediate host) during the time-of-day that cattle feed (Trail 1980).

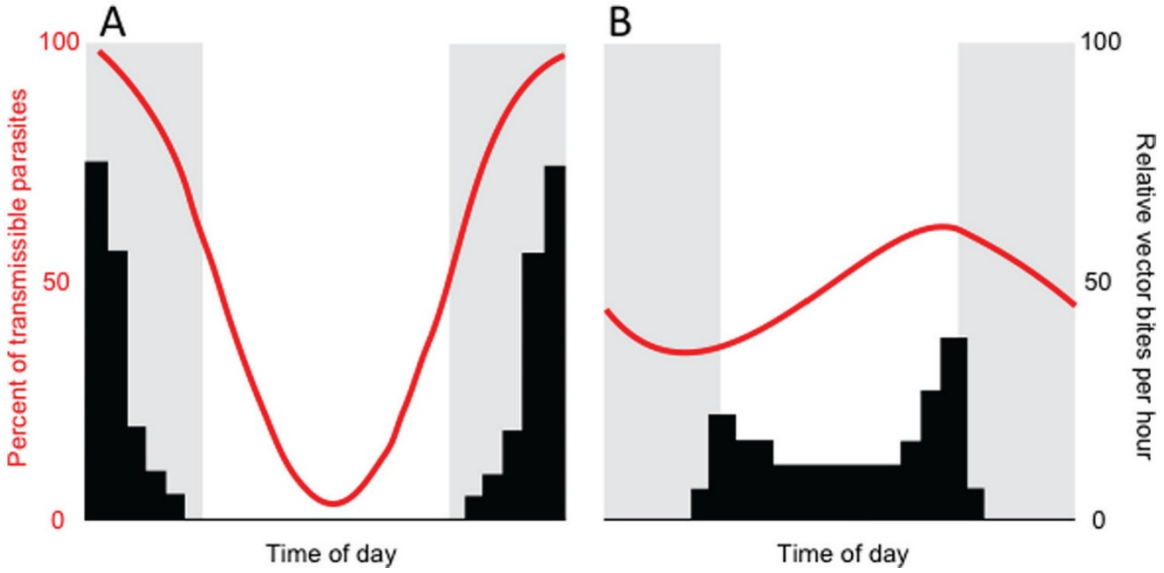

Figure 1: The migration of microfilariae from the lungs to the host's peripheral circulation broadly coincides with the activity rhythms of their mosquito vector species. Red lines illustrate rhythms in the percentage of the maximum number of microfilariae observed in the peripheral blood of hosts, and the bars illustrate vector biting activity. (A) The nocturnally periodic form of *Wuchereria bancrofti* is transmitted by night-biting *Anopheles* and *Culex*, and (B) the diurnally subperiodic form is transmitted by day-biting *Aedes*. Coinciding migration with vector foraging is thought to maximize parasite transmission, the "Hawking hypothesis." Figure taken from Reece et al (2017) and originally adapted from Pichon and Treuil (2004).

Malaria parasite rhythms

In the vertebrate host, malaria parasites (*Plasmodium* spp.) exhibit rhythms in the cycle of replication they undertake in red blood cells, termed their intraerythrocytic development cycle (IDC). The IDC is characterised by the changing forms of asexual stages as they progress through their development. The cycle begins with infection of new red blood cells by merozoites stages, these develop into ring stages, then into trophozoite stages of increasing size, finally becoming schizonts that each contain 8+ daughter parasites which eventually burst out of their red blood cells; 'schizogony'), releasing their progeny merozoites to start the cycle anew (Figure *2*a). *My thesis asks how are these rhythms controlled and why do they occur?*

The IDC duration varies between malaria species but durations are often multiples of 24 hours suggesting that it has a circadian basis e.g. the rodent malaria species *P. chabaudi* has a cycle duration ~24h and the most deadly human infecting species *P. falciparum*, has a 48h cycle (Mideo et al. 2013) (Figure *2*b). For most species of *Plasmodium* studied to date, parasites progress through the IDC in synchrony with each other (Figure *2*c). In synchronous infections, the inflammatory response associated with the simultaneous mass bursting of RBCs during schizogony causes a characteristic fever in humans which is so distinct and regular it was historically used as a diagnostic feature of the disease (Garcia et al. 2001) (in mice this manifests as paroxysm, a periodic chill). In contrast, some malaria species are

asynchronous with each parasite progressing through the IDC on a different schedule to others in the cohort (Figure *2*c). For asynchronous species, IDC duration can be estimated through artificial synchronization, and is generally shorter than 24 hours (22-23h for *P. berghei* and 18h for *P. yoelii)* (Mons et al. 1985, Gautret et al. 1995, Janse et al. 1995, Deharo et al. 1996). It is not known what aspect of their biology leads to a species being synchronous or asynchronous, nor whether asynchrony is a constraint or an alternative strategy to cope with the challenges of living in a rhythmic within-host environment (*addressed in paper #5*).

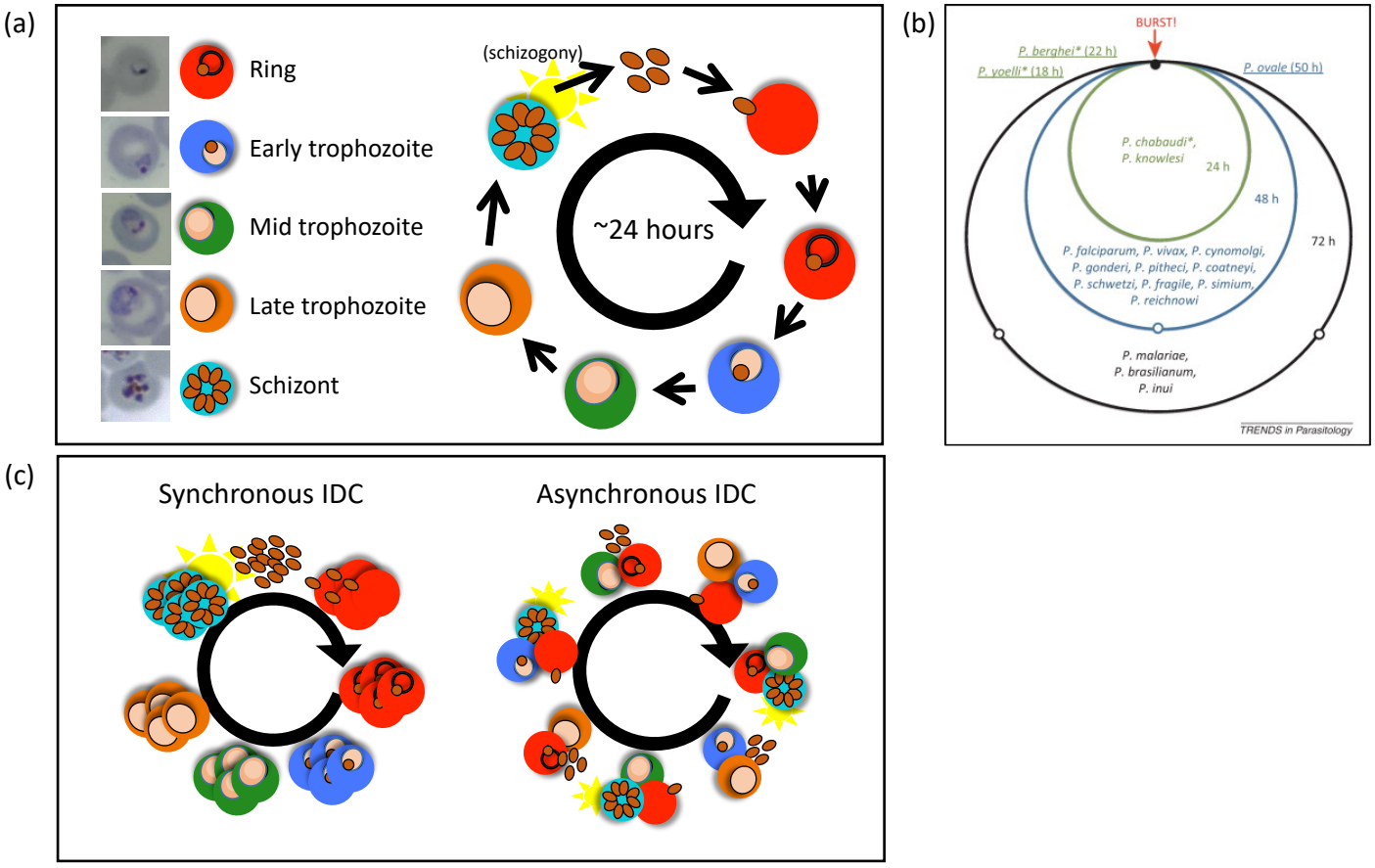

Figure 2: (a) The intraerythrocytic development cycle (IDC) consists of sequential development through a number of parasite stages: merozoites (no microscopy image provided) invade red blood cells, develop into rings, then transition into several trophozoite stages to become schizonts which burst and release merozoites that start the cycle anew. For the parasite species pictured, *P. chabaudi*, the IDC duration is approximately 24 hours. (b) The IDC durations of other malaria species are often multiples of 24 hours (Mideo et al. 2013). (c) Parasite development can be classed as synchronous, in which all parasites in the infection progress through the IDC in synchrony, or classed as asynchronous, in which each parasite in the infection is progressing through the IDC on a different schedule to others.

How are the rhythms of malaria parasites controlled?

Over 57% of the *P. chabaudi* genome exhibits 24hr periodicity (Subudhi et al. 2020) but to what extent malaria parasites are in control of this rhythmicity is unclear. The rhythmicity of human malaria parasites breaks down readily *in vitro* (Schuster 2002), as do the IDC rhythms of rodent malaria species at the peak of infection when the host's symptoms are the most severe (Prior 2018, O'Donnell et al. 2021), and IDC duration can be slowed by a reduction in temperature (Rojas et al. 1993). No homologs to the known clock genes from cyanobacteria, fungi, insects or mammals have been identified in *Plasmodium,* however gene expression patterns of both human and rodent malaria species display some hallmarks of an endogenous clock (Rijo-Ferreira et al. 2020, Smith et al. 2020, Subudhi et al. 2020). Malaria parasites are thought to meet two of the three criteria for possessing an endogenous oscillator as they persist in clock knockout mice (i.e. rhythms are capable of 'free-running') and are entrainable to host feeding-fasting schedules but as yet it has not been demonstrated that parasite rhythms are temperature compensated. Additionally, it is difficult to interpret if parasites are indeed 'free-running' in clock knockout mice because natural variation in the IDC duration has not been quantified. It may take several cycles for a rhythm to break down since parasites grow exponentially and passively reinforce their own rhythms by an influx of parasites post schizogony (Greischar et al. 2019). Parasites may also be constrained to a close to 24hr development period due to the physical mechanics of their development (e.g. if schizonts don't actively burst, their membranes will breakdown and they burst anyway). To what extent parasites have control over their IDC duration and synchrony is discussed in *paper #6*, however, strong evidence for parasite control of their IDC rhythm comes from knockout of the serpentine receptor gene SR10, which causes the IDC to become 2-3 hours shorter than the IDC of wild type parasites (Subudhi et al. 2020).

Without identifying a Zeitgeber for a malaria parasite clock or demonstrating temperature compensation, it is premature to assume they possess an endogenous circadian oscillator. Instead, parasites may keep time using a more rudimentary mechanism such as an 'hourglass timer' in which a timer is triggered following the detection of a time cue that stimulates, for instance, the parasite to transition between IDC stages. This would allow parasites to keep time but would not generate self-sustaining oscillations (Pittayakanchit et al. 2018). Alternatively, an even simpler strategy would be for parasites to use phenotypic plasticity to perform IDC transitions in direct response to the appearance or disappearance of time cues (i.e. stop/go triggers). Malaria parasites have demonstrated adaptive phenotypic plasticity in their ability to modify investment into sexual stages and alter their sex ratio in response to cues for changes in host health, coinfection with conspecific genotypes, and anti-malarial drugs (Reece et al. 2008, Schneider et al. 2018). The IDC schedule is plastic; it has long been known that following perturbation of the phase of the IDC relative to the phase of the host, the IDC readily recovers its normal schedule. Early literature describes *P. cathemerium* infections of canaries initiated with parasites whose IDC had been slowed down (through cooling *in vitro*) as speeding up once injected into a new host (Taliaferro 1928). Further, parasites in experimental animals entrained to a non-24 hour day (e.g. 14h light: 14h dark) had IDCs closer to 28 than 24 hours (Taliaferro 1928, Boyd 1929). These early experiments suffered many confounders but recent similar experiments with *P. chabaudi* (*papers #1, #2, #4 and #6*) also show that the IDC schedule recovers from a 12 hour temporal misalignment within 5-7 cycles, and rescheduling occurs by speeding up the IDC.

Whilst recent work suggests parasites are at least in part in control of their IDC schedule, early work on the topic assumed that parasites are intrinsically arrhythmic and allow host rhythms to schedule them.

Specifically, because IDC stages vary in their tolerance to heat shock during fever caused by schizogony, rhythmic bouts of fever generate a daily window in which certain IDC stages are selectively killed, thus, generating synchrony and timing of the IDC (Kwiatkowski 1989). However, there is circular logic in this hypothesis because the fever that supposedly enforces rhythmicity only occurs when parasites are already rhythmic; so it doesn't explain how parasites have become sufficiently rhythmic to generate fever in the first place (Reece et al. 2017, Prior et al. 2020). Furthermore, if rhythms are established by the killing of mis-timed IDC stages, the death of a large number of parasites would be expected in the first cycles following experimental perturbation of the time (phase) of the IDC. However, in contrast, mismatch between host and parasite rhythms only seems to cause modest costs (*addressed in papers #1, #2, #4 and #6*).

Unlike the blood-borne parasite *Trypanosome brucei*, malaria parasites do not use host temperature rhythms to schedule the IDC (Rijo-Ferreira et al. 2017), nor do they use light:dark cycles (Prior et al. 2018, O'Donnell et al. 2020). Malaria parasites in hosts that were housed in the same light:dark cycle but had opposite feeding-fasting schedules (i.e. day fed vs night fed) aligned their IDC schedule with the phase of the host's feeding-fasting rhythm. Specifically, aligning the start of schizogony in the second half of the feeding window; around midnight for rodents (Prior et al. 2018). In this experiment, host locomotor activity and temperature rhythms were not fully inverted between day- and night-fed mice, allowing locomotor activity and body temperature to be ruled out as drivers of the IDC schedule and glucose to be proposed as a time cue/Zeitgeber (Prior et al. 2018). Further, timing cues from feeding-fasting rhythms may be amplified as infections progress because parasites stimulate the production of the inflammatory cytokine TNF-α, which increases the energy demands of immune cells and causes hypoglycemia during the rest phase (thus making the glucose nadir lower) (Hirako et al. 2018). In keeping with these findings, the IDC rhythm dampens in mice that are diabetic or deficient in TNF-α (Hirako et al. 2018). However, more direct tests fail to support a role for glucose rhythms as a time cue. Instead, isoleucine has emerged as sufficient to provide a time-of-day cue/Zeitgeber for parasites to align the phase of the IDC with host feeding (Prior et al. 2021). Isoleucine is the only amino acid present in food that parasites cannot readily scavenge from the digestion of haemoglobin (Prior et al. 2021). For both rodent and human malaria species, withdrawal of isoleucine *in vitro* leads to IDC dormancy and upon replenishment of isoleucine, parasites restart development with no ill effects (Babbitt et al. 2012, McLean et al. 2020, Prior et al. 2021). In contrast, withdrawal of other essential resources (e.g. glucose) results in rapid starvation responses and parasite death (Babbitt et al. 2012).

Why are malaria parasites rhythmic?

The observation that an aspect of the host's feeding-fasting rhythm sets the IDC schedule fits with the most energetically demanding parasite stages (late trophozoites and schizonts) occurring only during the host's feeding period. As well as being a limited resource for malaria parasites, isoleucine may also act as a host timing signal for the availability of vitamins, cofactors, purines, folic acid, pantothenic acid, and glucose, that are perhaps more economical for the parasite to scavenge rather than synthesize themselves (Prior et al. 2021). Aligning to the host's feeding-fasting rhythm may be particularly important when parasites are at high densities and resources are their most limited. If so, it would be important for parasites to be in the correct schedule before reaching high densities, which may explain the modest costs of mismatch to parasites observed early in the infection (*paper #2*). Further, part of the host's sickness response is lethargy and reduced feeding (Hart 1988) and this breakdown of feeding-

fasting rhythms and absence of a reliable host timing cue may explain why parasites lose synchrony during the peak of infection.

Whilst recent work points to within-host survival being maximized by coordinating particularly resource-hungry IDC stages with the phase of host feeding/ digestion, avoiding clearance by the spleen is a potentially additional benefit of scheduling the IDC. A feature of many malaria parasite species is their ability to escape capture and clearance when circulating through the spleen by cytoadherence of the infected RBCs within tissues (such lung or adipose tissue). Sequestration occurs in late IDC stage parasites, which appear in the evening in *P. chabaudi* infections, which is also the time-of-day that macrophages are most active in mice (Franke-Fayard et al. 2010, Scheiermann et al. 2013). Thus, perhaps host feeding-fasting rhythms also provide a proxy for rhythms in how dangerous the spleen is. There is extensive research on the mechanics of binding to tissues by infected RBCs, but whether sequestration is particularly important at a certain time-of-day, has been overlooked. Alternatively, sequestration might be equally beneficial throughout the circadian cycle, but rhythms in host binding receptor availability dictate when parasites can sequester.

Instead of focusing on within-host survival, early studies attempting to explain IDC rhythms were inspired by Hawking's observations of *Wucheria spp.* (Hawking 1967) and proposed that the IDC schedule sets the phase of when transmission stages become infective to vectors. During each IDC, a small proportion of parasites do not replicate asexually, but become sexual stages (gametocytes), which are responsible for transmission. When an insect vector takes a blood meal from an infected host, gametocytes rapidly produce gametes, mate, and the zygote stage (ookinete) begins the developmental processes and replication required to produce stages that are infective new hosts (sporozoites)(Figure *3*). Hawking proposed that schizogony determines when gametocytes begin their development and should be scheduled such that gametocytes reach sexual maturity at the time-of-day vectors forage for blood (Hawking et al. 1968, Hawking 1970).

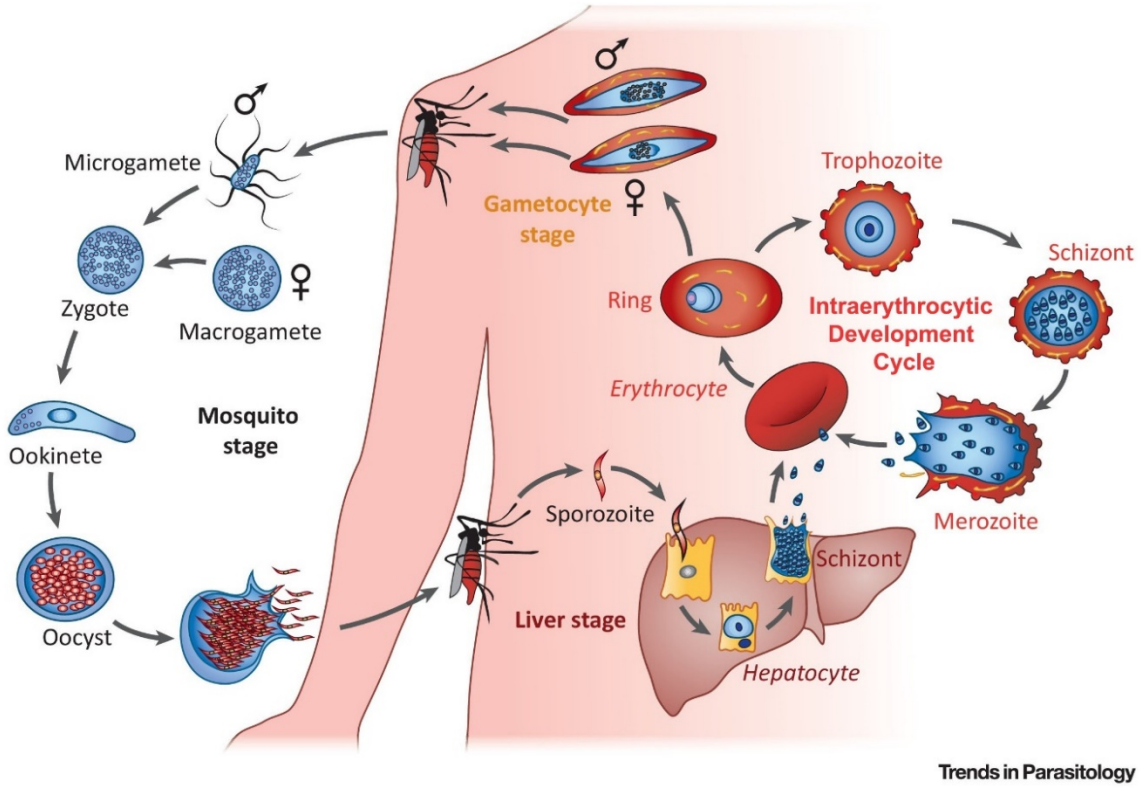

Figure 3: The life cycle of malaria parasites (*P. falciparum* illustrated) consists of development in two hosts. Sporozoites enter the vertebrate host from a mosquito bite. After initial growth inside liver cells, 1000s of parasites are released into the blood stream where they invade red blood cells and begin the intraerythrocytic development cycle (IDC). At each round of the IDC a proportion of parasites commit to becoming gametocytes (sexual transmission stages). Gametocytes are taken up in the mosquito blood meal where they mate and form growth stages (oocysts) within the mosquito midgut wall. Sporozoites, released from oocysts, migrate to the salivary glands where they will be injected into a new host and continue the cycle.

Tests of Hawking's hypothesis have given conflicting conclusions, including: (i) Across several species of *Plasmodium*, differentiation into gametes by males and parasite burdens within mosquitos were higher when transmissions occurred at the time-of-day their vector is most active compared to the day time (Garnham et al. 1974). (ii) Transmission to mosquitoes was more successful at night compared to in the day time for an avian malaria species (*P. relictum*) with an arrhythmic IDC (Pigeault et al. 2018). (iii) In the host's blood, gametocyte densities of *P. falciparum* peak during the day time, at the opposite phase to peak mosquito activity (Magesa et al. 2000). (iv) Parasite burden within mosquitoes following infection with either *P. falciparum* or *P. chabaudi* did not differ between day and night transmissions (Bray et al. 1976, Githeko et al. 1993, Gautret et al. 1996). (v) For *P. vivax*, which transmits via nocturnally active mosquitoes, parasite burden within mosquitoes was higher following day feeds (Karunaweera et al. 1992). (vi) Precise timing is likely to be only relevant to species with fast developing and short lived gametocytes like *P. chabaudi* for which gametocytes reach maturity within 48 hours and have an estimated lifespan of ~20 hours rather than 'long-lived' species such as *P. falciparum* for which gametocytes take 10 days to reach maturity and live for a similar time frame.

A potential explanation for these contradictory findings is the failure to account for any roles of host and vector rhythms in transmission (*addressed in paper #3*). Mosquitoes exhibit their own biological rhythms including rhythms in immune gene expression and activity (e.g. melanization) that may directly shape how susceptible they are to new infection (Kumar et al. 2003, Rund et al. 2013, Rund et al. 2016). Many important components of the host immune system are also rhythmic (Scheiermann et al. 2013, Scheiermann et al. 2018) and these may also influence transmission (Rund et al. 2016). The rhythms of the vector and the host may act synergistically or antagonistically to each other and to the parasite's rhythms. For instance, if rhythms in mosquito susceptibility and parasite infectivity oppose each other they may cancel each other out, eroding the ability to detect the underlying time-of-day effects (e.g. transmission may happen when mosquitoes are most susceptible and parasites are least infective). Indeed, recent study that separates parasite and mosquito rhythms reveals that at night, gametocytes are at a lower density in the host's blood but are twice as infective as day time gametocytes, and that parasite burden is 4 fold higher in mosquitoes that become infected in the day time (Schneider et al. 2018). These rhythms become exacerbated as parasites progress through their development within mosquitoes, resulting in far more host-infective stages in the salivary glands when night time parasites infect day time mosquitoes (Schneider et al. 2018).

Taking all these observations together suggests that the IDC schedule brings the dual fitness benefits of maximizing within-host survival and between-host transmission (Prior et al. 2020). By getting its timing right, *P. chabaudi* appears able to exploit the resources it requires from the host's food as well as capitalize on vector rhythms by producing the most infective gametocytes when the opportunity to transmit arises. However, whether exactly the same IDC schedule maximises the benefits of coordinating with host and vector rhythms is unclear. Perhaps there is a convenient coincidence for the parasite here, or perhaps the benefits of aligning with host feeding-fasting rhythms is the primary selective force for the IDC schedule and the duration of gametocyte development has been selected on to fit in with this timing.

Why care about malaria parasite rhythms?

Understanding the IDC schedule of malaria parasites is important because successive cycles of asexual replication are responsible for disease symptoms of malaria (Gazzinelli et al. 2014) and fuels the production of gametocytes that are responsible for transmission (Carter et al. 2013). The balance of evidence suggests that parasites are in large part in control of the IDC schedule and use a time keeping mechanism to prevent natural variation in the IDC duration from eroding the alignment with host and vector rhythms. Given that the IDC schedule is beneficial to parasites, developing interventions that disrupt parasite time keeping could reduce both the severity of disease and transmission, providing benefits at the levels of the individual and the population (Prior et al. 2020).

Targeting the IDC schedule could also help make existing drug treatments more effective. For example, different IDC stages vary in their sensitivity to drugs so drugs could be more effectively used if they target the most vulnerable stage (Cambie et al. 1991), and this stage-specific vulnerability can be exacerbated by misalignment to host rhythms (Owolabi et al. 2021). Furthermore, interfering with parasite control of the IDC schedule could help in the fight against drug resistance. In response to adverse situations such as antimalarial drug treatments, ring stages slow their development to the point of dormancy (Teuscher et al. 2010). This confers the ability to 'wait it out' until the drug is metabolized by the host and it is safe to restart metabolism and development. Being able to pause or speed up the

IDC in stressful situations may have evolved to cope with periods of host sickness (Birget et al. 2019), or to ensure the erythrocytic phase of the infection readily aligns with host rhythms following arrhythmic emergence from the liver phase of the life cycle. Thus, understanding how parasites organize the IDC schedule could prevent them from using dormancy to tolerate drugs. By timing drug treatment according to when it is most effective against parasites, it might be possible to administer lower doses which makes drugs less toxic to hosts (Cambie et al. 1991, Baraldo 2008). It might also be possible to harness host rhythms to enhance drug efficacy. For example, the half-life of drugs could be extended by giving treatments in the rest phase (when metabolism is slower) to prolong the window they can act on parasites. Alternatively, drugs that need to be metabolized into an active form may be more effective if administered in the host's active phase.

In addition to exploiting IDC rhythms as a target for interventions in the host, a better understanding of the ecology of parasite rhythms is important for preventing transmission. There is mounting evidence that mosquitoes are altering the timing of their host-seeking and biting behaviours in response to the use of insecticide treated bed nets (Yohannes et al. 2012, Sougoufara et al. 2014, Cooke et al. 2015, Wamae et al. 2015). Specifically, to evade insecticides and acquire blood meals from unprotected hosts, mosquitoes are biting earlier in the evening or further into the morning. However, the consequences of altered vector rhythms for malaria control are unknown (Thomsen et al. 2017), but are likely to involve an increase in transmission opportunities. Whether parasites can fully exploit this opportunity may depend on how much flexibility there is in the IDC schedule, or in the developmental duration of gametocytes, to keep up with changes to vector timing, as well as the consequences of temporal misalignment between blood feeding and other clock driven processes for mosquito fitness and population dynamics (*addressed in paper #3*).

Aims & objectives

In my collected first author published works, I use a rodent malaria model system to explore the interactions between parasites and their hosts and vectors. My portfolio spans 10 years of research, from initial investigations to verify that the IDC schedule is adaptive within the host before asking questions about its role in transmission and how the IDC schedule is established. By applying a multidisciplinary approach incorporating chronobiology, evolutionary ecology, parasitology, and entomology my research has advanced understanding of the 'What?', 'How?', and 'Why?' of malaria chronobiology by asking the following:

i. **Are there fitness benefits to malaria parasites of having an IDC schedule temporally aligned with the host's rhythm(s)?**
O'Donnell A. J., Schneider P., McWatters H. G. and Reece S. E. (2011). "Fitness costs of disrupting circadian rhythms in malaria parasites." Proc. Royal Soc. B 278: 2429 - 2436.

ii. **Are the consequences of temporal mis-alignment to host rhythms specific to a particular IDC stage or route of infection?**
O'Donnell, A. J., Mideo N. and Reece S. E. (2013). "Disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated." Malaria Journal 12: 372. And O'Donnell, A. J., Mideo N. and Reece S. E. (2014). "Correction: disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated." Malar. J. 13(1): 503.

iii. **What are the consequences of altering the time-of-day of infection, for malaria parasite transmission and mosquito life history traits?**
O'Donnell, A. J., Rund S. S. C. and Reece S. E. (2019). "Time-of-day of blood-feeding: effects on mosquito life history and malaria transmission." Parasites Vectors 12(1): 1-16.

iv. **Are rhythms driven by the host's canonical transcription-translation feedback loop clock responsible for the IDC schedule?**
O'Donnell, A. J., Prior K. F. and Reece S. E. (2020). "Host circadian clocks do not set the schedule for the within-host replication of malaria parasites." Proc. Royal Soc. B 287(1932): 20200347.

v. **Does the ecology of IDC rhythms in synchronous species of malaria parasite extend to asynchronously replicating species?**
O'Donnell, A. J. and Reece S. E. (2021a). "Ecology of asynchronous asexual replication: the intraerythrocytic development cycle of *Plasmodium berghei* is resistant to host rhythms." Malar. J. 20(1): 1-12.

vi. **How do malaria parasites become realigned with host rhythms following temporal misalignment?**
O'Donnell, A. J., Greischar M.A. & Reece S. E. (2021b). "Mistimed malaria parasites re-synchronise with host feeding-fasting rhythms by shortening the duration of intra-erythrocytic development." Parasite Immunol. e12898

Methodology

In all the experiments included in this work I use the rodent malaria model system (specifically *P. chabaudi* and *P. berghei*) instead of the human malaria model, *P. falciparum*, which has obvious ethical issues with regards to experimental work. While infections of *P. chabaudi* and *P. falciparum* differ in details, *P. chabaudi* is considered a good model for human malaria (Stephens et al. 2012). The bank of *P. chabaudi* genotypes provides a wealth of genetic variation, the raw material for natural selection, which can be used to test evolutionary theories. *P. falciparum* can be studied in culture, however, understanding how parasites interact with their environment i.e. the complex conditions parasites experience in the host, is not tractable *in vitro*. Working with lab reared mice (and tractable mosquito vectors) allows for more realistic parasite ecology, yet facilitates controlled and standardised conditions between experimental infections. Overall, the general evolutionary principles I test using *P. chabaudi* apply across the tree of life, thus what I discover in *P. chabaudi* likely extends to other species within the *Plasmodium* genus.

Parasites

All parasites were sourced from the Malaria Reagents Repository (www.malariaresearch.eu) at the University of Edinburgh. These parasites were initially isolated from wild tree rats during expeditions across central Africa throughout the 1960s-70s. Animals were brought to the lab and parasites were cloned, transmitted through mosquitoes, lineages tracked and clonal lines (genotypes) cryopreserved in liquid nitrogen until present day. Two parasite species feature in my portfolio: a synchronous malaria species *P. chabaudi (*subspecies *P.c. chabaudi* and *P.c. adami*), and *P. berghei,* an asynchronous species (Figure 2c). For all experiments, wild type parasites genotypes were used and were all within a maximum of 10 generations from a mosquito transmission event (because transmission regulates parasite virulence (Spence et al. 2013).

P. chabaudi originates from lowland forests of central Africa and genotypes with both subspecies display a spectrum of asexual replication rates, investment in sexual stage gametocytes, virulence to hosts (usually measured by weight loss and anaemia), propensity to sequester, and competitive ability. This variation enables investigation of the relationships between life history traits, parasite genetics, and environmental change. For example, genotype AJ (*P. chabaudi* chabaudi) replicates to a high density and exhibits a moderate level of virulence while DK (*P. chabaudi* adami) is far less virulent. Notably, these clones also vary by the degree by which they disrupt host rhythms in locomotor activity (lethargy) and body temperature (hypothermia) with AJ causing far more disruption to rhythms than DK (Prior et al. 2019). Therefore, my initial papers focused on AJ because its dynamics are readily quantified but later papers focused on DK because preserving the rhythmicity of hosts throughout infections was necessary.

A representative *P. chabaudi* infection is shown in Figure 4. Parasite replication is exponential and reaches a peak density between days 7-8 and, as a consequence of this, hosts experience a subsequent nadir in their red blood cell densities. This is followed by a window of host recovery during which asexual parasite density crashes and are only maintained at very low densities. Gametocyte dynamics lag behind asexuals with a large investment in transmission stages around day 15 PI (Figure 4a). In a synchronous infection, the IDC can be clearly observed across a time series with a peak of ring stages occurring at the end of the feeding period (ZT23) and the subsequent development into the various

trophozoite stages. Due to sequestration, late trophozoites and schizonts stages are rarely observed in the bloodstream but daily rhythms can still be detected (Figure 4b).

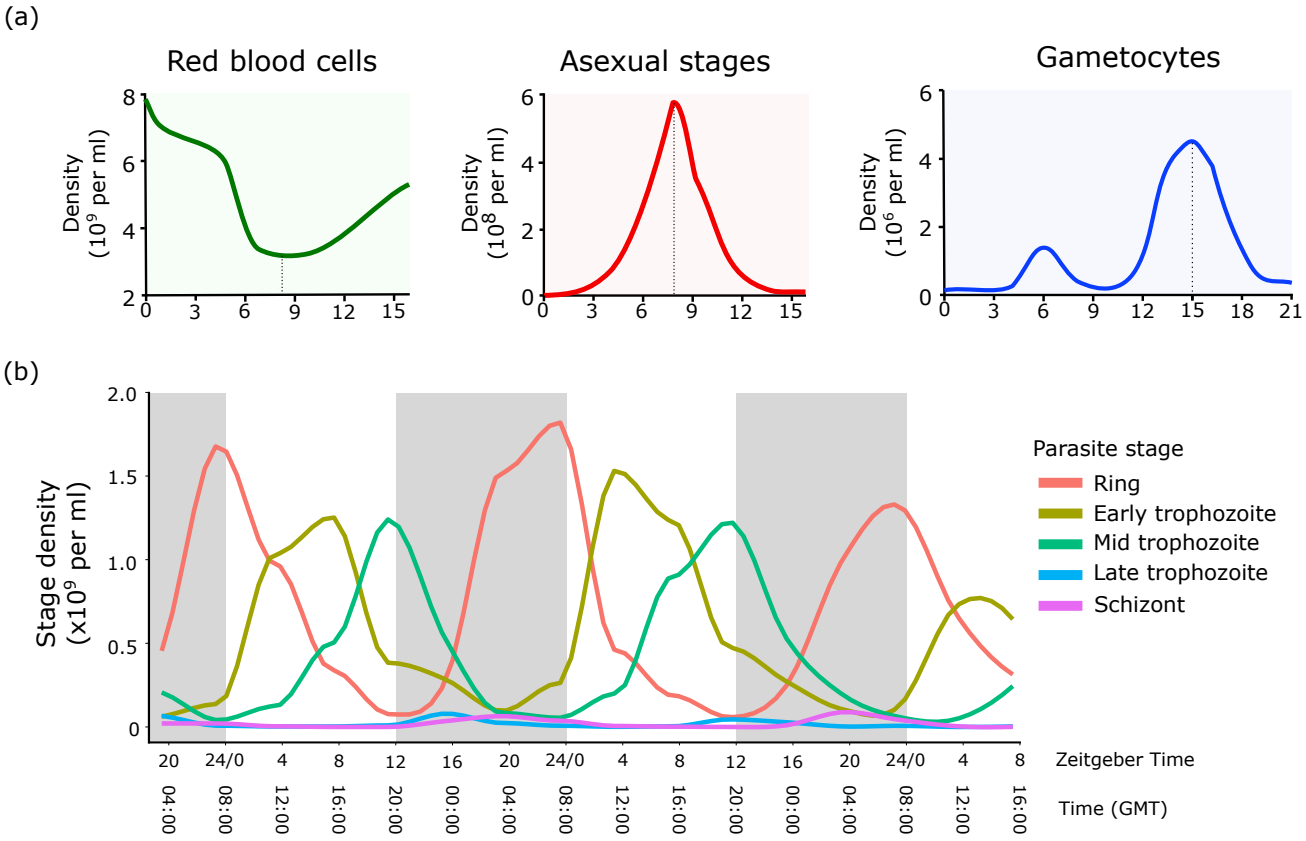

Figure 4: Dynamics of a typical *P. chabaudi* infection. (a) Illustrative host-health (red blood cell density) and parasite (asexual and gametocyte densities) dynamics. The dotted line on each panel highlights the day on which densities are at their lowest (red blood cell) or highest (parasite measures). (b) Stage densities from a synchronous infection demonstrating the sequential development of each parasite stage and the time-of-day they occur relative to the photo-schedule. Photo-schedule is indicated by shading (grey = lights OFF) and is analogous to the host's feeding window. Shown are densities at the peak of infection on days 7-9 post infection. The third peak in ring stage density occurs when overall parasite density is declining and hosts are recovering and thus is lower than previous peaks. Time is shown in both Zeitgeber time (hours relative to lights ON) and in GMT.

P. berghei ANKA originates from the cool African highlands of Katanga and is asynchronous (i.e. individual asexual stages complete their IDC at any time-of-day). Unlike *P. chabaudi* infections which exhibit acute infection for 14 days and then a chronic infection period that can last several weeks, *P. berghei* infections in the lab are short lived lasting only 6-8 days before the host dies from complications associated with cerebral malaria. As well as permitting questions to be asked about asynchronous asexual replication, *P. berghei* is regarded as the most tractable model for transmission studies because it readily infects mosquitoes with a high infection burden.

For both parasite species, controlled infections are initiated simply by taking infected blood from infected donors to inject a specific number of parasitized red blood cells (RBCs) intraperitoneally or intravenously into recipient mice. Parasites densities (per ml) are calculated using the product of parasitemia (proportion infected RBC) multiplied by RBC density (cells per ml). Blood is diluted (if necessary) with citrate saline (0.85% NaCl, 1.5% tri-sodium citrate) resulting in an inoculum with a precise dose (usually one or ten million infected RBCs per 100µl). Unlike the human malaria *P. falciparum,* neither *P. chabaudi* or *P. berghei* are adapted to long term *in vitro* culture but short term culture can be carried out to rear parasites within a single IDC, or to initiate mating and zygote development. Further details for the set up and sampling of infection are given in the methods sections for each paper in the portfolio.

Hosts

The natural mammalian hosts for *P. chabaudi* and *P. berghei* are thicket rats (*Grammomys sp.*) which are very difficult to rear in the lab. Instead, decades of malaria research has been conducted using the model lab mouse (*Mus musculus*). Being a model species, lab mice are far more tractable than thicket rats and well established methods are available for quantifying and perturbing their physiology (including immune responses, anaemia, and rhythms). My portfolio uses three strains of lab mouse: MF1, an outbred stock chosen for their large size (~40g) and tolerance to malaria infection; C57BL/6JCrl (C57BL/6) an inbred strain that are smaller than MF1 but are more widely used in behavioural studies and provide a wild type control for the third strain of *Per1/2*-null mice. Derived from a JAX mouse strain *Per1/2*-null mice have been backcrossed onto a C57BL/6 background for over 10 generations. *Per1/2*-null mice do not have the TTFL clock genes Period1 and Period2 and are behaviourally and physiologically arrhythmic (e.g. no feeding, temperature and locomotor rhythms) when placed in constant conditions, such as constant darkness (Bae et al. 2001, Maywood et al. 2014).

To maintain ecological realism of host biology, experimental mice were group housed where possible (though in *paper #4* single housing was necessary) and were kept at ~21C in rearing rooms or rearing cabinets (Scantainers) with fluorescent/LED lighting. Changing the light schedule between cabinets/rooms allows infections to be compared across the equivalent of different time zones e.g. lights on at 0700 GMT; lights off at 1900 GMT (LD) versus the reversed schedule of lights on at 1900 and lights off at 0700 (DL). In cases where mice are housed in constant darkness conditions (DD) a very dim (<10 lux) red LED was used to allow researchers to monitor and sample mice (it is thought that dim red light does not influence mouse sleep wake cycles (Zhang et al. 2017)). After any manipulation of photo-schedule, mice were given at least 14 days to acclimatize (entrain) to their new schedules before experimental infections began.

Feeding-fasting schedules also varied within and between experiments. In most experiments mice were allowed to feed *ad libitum*, but in some cases where timing of feeding and fasting is a core experimental manipulation (*papers #4-6*) a time restricted feeding schedule (TRF) was applied in which food was only available to mice for 10-12 hours each circadian cycle (by changing food hoppers and manually sweeping the cage for stray food pellets whenever access to food was given/removed). In some cases TRF was applied to *Per1/2*-null mice to generate rhythms in processes associated with feeding-fasting and in other cases TRF was used to disrupt the temporal alignment between food (peripheral) and light-driven clocks. In cases where animals are fed *ad libitum* (i.e. all day fed) the actual feeding-fasting rhythms are dependent on the state of the mouse's endogenous clock. For example, when housed in LD wild type

mice feed naturally in their dark period, and this schedule continues (free runs) in constant darkness. Specifically, for mice, the endogenous clock free runs slightly shorter than 24 hours (around 23.5-23.8h for C57Bl/6), so feeding begins slightly earlier each 24h period. For all-day fed *Per1/2*-null mice housed in constant conditions (DD), feeding is arhythmic with repeated short (10-30mins) bouts of feeding across the whole 24 period. After any manipulation of feeding-fasting rhythms, mice were given at least 10 days to adjust or entrain to their new schedules before experimental infections began. TRF protocols differ from dietary restriction in that there are no weight loss implications of TRF. Further details of the perturbations applied to hosts are given in the methods sections for each paper in the portfolio.

Vector

The natural vector is not known for *P. chabaudi* but has been identified for *P. berghei* (*Anopheles dureni millecampsi,* which is shared with another rodent malaria species, *P. vinckei vinckei)* but both *P. berghei* and *P. chabaudi* transmit readily to the generalist mosquito, *Anopheles stephensi*. *An. stephensi* is an important vector for malaria in India and one of the few malaria vectors that is well adapted to an urban environment. Recent work has suggested that this vector has expanded its range in India and appearing in many populated regions of Africa (Sinka et al. 2020). *An. stephensi* are active primarily in the evening with a peak in activity at the start of the evening (Korgaonkar et al. 2012). The *An. stephensi* at the University of Edinburgh originated in Pakistan are readily reared in the lab at 26C, 60-70% humidity and are fed 8% fructose solution to mimic nectar, a natural source of sugar. During transmission, mosquitoes are housed at 26C for *P. chabaudi* or at 21C for *P. berghei* (reflecting the cooler climate of gallery forests that are the natural habitat for *P. berghei*). It is not tractable to sample parasites within mosquitoes, during mating and ookinete development, but destructive sampling and quantification of later stages on the midgut (oocyst) and salivary glands (sporozoite) is accurate.

Experimental designs

Many of the experiments in the portfolio are designed to probe aspects of alignment between host and parasite rhythms, so follow a similar approach (Figure 5, Table 1). Parasites are expanded in wild type donor mice that are housed in one of the same photoperiod schedules as an experimental group. When the donor infections reach a threshold in asexual density, parasites are harvested and used to simultaneously infect the experimental groups. Parasites that move from donors to experimental mice entrained to the same photo-schedule are considered temporally aligned (matched) to the host rhythm because the parasite IDC is on the 'correct' schedule for the new host. In contrast, when parasites move to experimental mice entrained to the opposite photo-schedule to the donors, parasites instantaneously become mismatched to their new host 12 hours (Figure 5).

Experimental infections are sampled frequently to compare how the IDC schedule behaves in the different treatment groups. Sampling regimes range from daily or twice daily sampling to intensive 4 hourly sampling over 24-36 hours. Such intensive sampling for more than 48 hours directly affects the health of mice, including affecting their rhythms which has knock on unintended consequences for the parasite's rhythm. Thus, when a long duration of intensive sampling is required, several cohorts of experimental mice are infected and each group contributes samples for a series of sequential 32 hour windows. By concatenating data across cohorts, a multi-day-time-series is generated for each treatment group.

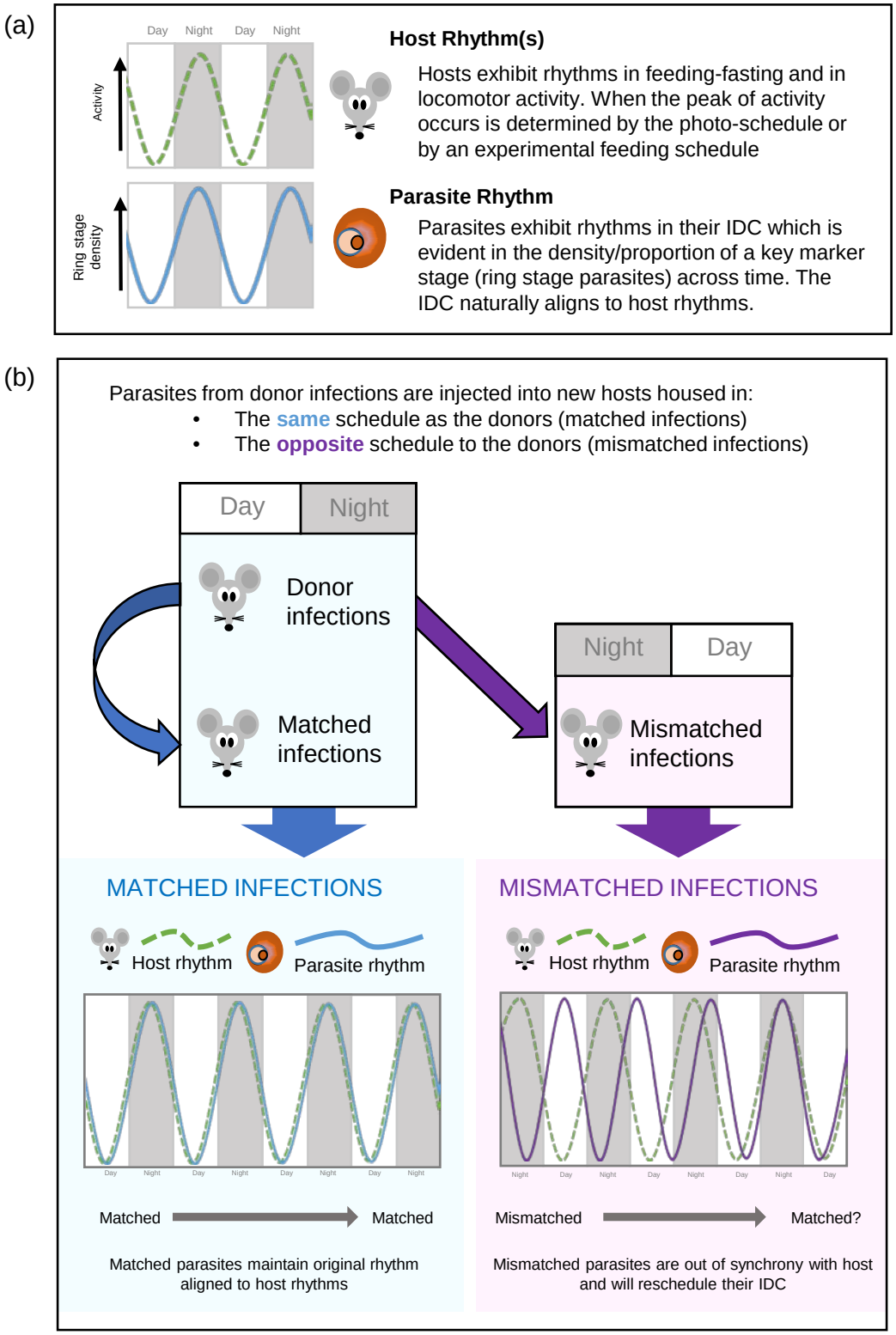

Figure 5: (a) Hosts exhibit behavioral rhythms that are driven by their internal clock. Parasites also exhibit rhythms in their intraerythrocytic development cycle (IDC), for example, shown are the daily rhythms in the density of ring stage parasites. In a control infection, parasite rhythms are in alignment with host rhythms. (b) Experimental design. Parasites are grown in donors under a particular photo or feeding-fasting schedule and these parasites are used to generate experimental infections in new hosts. Parasites remaining in the same schedule are matched to host rhythms and act as controls. Parasites transferred between schedules to hosts that are on the opposite rhythm to the parasite donor are temporally mismatched and must reschedule to regain synchrony with the host. Shaded boxes represent lights off (night) and for nocturnal mice are analogous to the host's feeding window.

Quantifying host and parasite rhythms

Circular rhythms that repeat each day, such as the IDC schedule can be represented by a curve over time. One such way to detect a rhythm is to simply fit a curve to the data using harmonic regression (Fourier analysis) and determine if the curve explains the data better than a straight line (at the mean) via an F-test (Stroebel et al. 2010). However, alternative methods that detect rhythms without curve fitting also exist including the stochastic modelling approach Maximum Entropy Spectral Analysis (MESA, (Dowse 2013)) that applies autoregressive models to the data (*this method is used in paper #6*). For both approaches, once a rhythm has been detected the parameters of the rhythm, phase, amplitude, and period, can be interpreted and compared between experimental treatments.

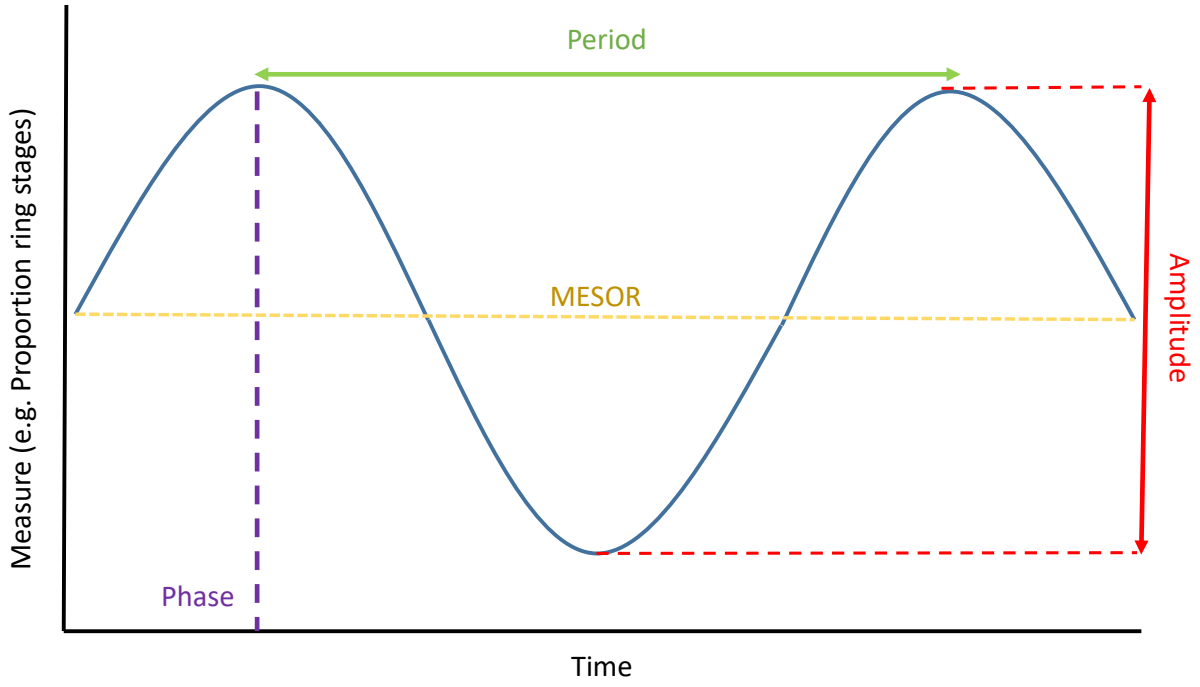

Figure 6: The parameters of a rhythm. The mean value of the curve is represented by the MESOR (midline estimating statistic of rhythm; in yellow). The amplitude (red) is the difference between peak and the nadir of the rhythm (i.e. twice the distance from peak or nadir and the MESOR). The phase (purple) is the timing of the peak of the rhythm. The period (green) is the time duration for the rhythm to re-occur.

"Phase" (in purple in Figure 6) is the timing of a reference point in the cycle such as the first peak (the acrophase) relative to a Zeitgeber (e.g. time of lights on) or the beginning of the sampling period. In the context of the IDC, peak refers to the time each day at which the peak of the focal IDC stage occurs. Over the period my portfolio covers, the proportion of asexuals at the ring stage has emerged from multiple labs as the best phase marker for the IDC (Hirako et al. 2018, Prior et al. 2018, O'Donnell et al. 2020, Rijo-Ferreira et al. 2020, O'Donnell et al. 2021).

Two 24h rhythms with phases that are 12 hours apart (e.g. the IDC rhythms of parasites in hosts entrained to standard and reversed photoperiods) will be in the inverse of each other (i.e. the peak of

one rhythm coincides with the trough for the other). Similarly, if the two rhythms were in fact two states of one individual changing across time then the difference in phase between the two rhythms is called a phase-shift. When an infection undergoes a phase-shift in response to a perturbation of the host's rhythm the IDC 'reschedules' with a phase shift over successive IDCs.

"Amplitude" (in red in Figure 6) is a unit-less measure between 0 and 1 representing the distance between the peak and the trough. In the context of parasite data such as the proportion of asexuals at the ring stage, amplitude is a proxy for how synchronous the cohort is. A highly synchronous species will have an amplitude close to 1, meaning that when ring stages peak, almost all asexuals are at this stage, and almost none of the parasites are at the ring stage when the rhythm hits its trough. The lower the amplitude, the damper it is, with 0 representing a flat line. Amplitude is calculated around the midpoint (the mean) of the rhythm, which is the MESOR (in brown in Figure 6).

"Period" (in green in Figure 6) is the duration of time taken for the cycle to repeat (e.g. from sunrise to sunrise is approximately 24 hours). For parasites, I use period as the estimate for the IDC duration. This measure can be difficult to calculate and several rhythm fitting algorithms are specifically designed to calculate period from different kinds of data, with some performing best when determining period from short datasets (harmonic regression) and others are better at determining period in rhythms with large changes in amplitude (MESA), therefore it is good practice to use multiple disparate methods as a form of validation.

Paper #	Year of Publication	Host Strain(s)	Parasite species	Parasite genotype	Parasite IDC	Experimental manipulation(s)	Parasite stage injected	Route of injection	Sampling
1	2011	MF1	*P. chabaudi*	AJ	Synchronous	• Matched vs Mismatch	Rings	IP injection	Daily (days 1-7 PI)
2	2013/2014	MF1	*P. chabaudi*	AJ	Synchronous	• Matched vs Mismatch	Rings & Trophozoites	IP and IV	Daily (days 1-7 PI)
3	2019	MF1 C57/Bl6 *Per1/2*-null	*P. berghei*	ANKA	Asynchronous	• Blood meal (Mosquito) time-of-day • Blood meal quality • Host time-of-day • *P. berghei* infection	Asynchronous mix	Mosquito transmission	Various
4	2020	C57/Bl6 *Per1/2*-null	*P. chabaudi*	DK	Synchronous	• Arrythmic vs Rhythmic host feeding	Rings / Asynchronous mix	IV injection	Time series: (4 hourly; day 5-6 PI)
5	2021a	MF1	*P. berghei*	ANKA	Asynchronous	• Matched vs Mismatch • Aarrythmic vs Rhythmic host feeding	Asynchronous mix	IP and IV	Daily: (days 2-6 PI) Time series: (4 hourly; day 5-6 PI)
6	2021b	C57/Bl6 *Per1/2*-null	*P. chabaudi*	DK	Synchronous	•Matched vs Mismatch •Arrythmic vs Rhythmic •host feeding	Rings	IV injection	Multi Day Time series (4 hourly; day 2-11 PI)

Table 1: Summary of the host-parasite combinations used, experimental manipulations, design and sampling schedule for each paper in the portfolio.

Results

The experiments summarized in Table 1 were designed to determine what the characteristics of the IDC rhythm are ("what") under normal conditions, how the IDC schedule is established in terms of which host rhythms influence it and how it responds to perturbation ("how"), and whether the IDC schedule is beneficial to parasites ("why").

What?

The ability to explain the IDC schedule first rests on an accurate description of its fundamental features. Ring stage rhythms from a typical matched infection (Figure 7) will have a period around ~24 hours with an amplitude ranging from 0.75-0.96 and peak ring stage parasite phase occurring just before lights ON at ZT23 (~ 11 hours after the start of host feeding) (*papers #4 & #6*).

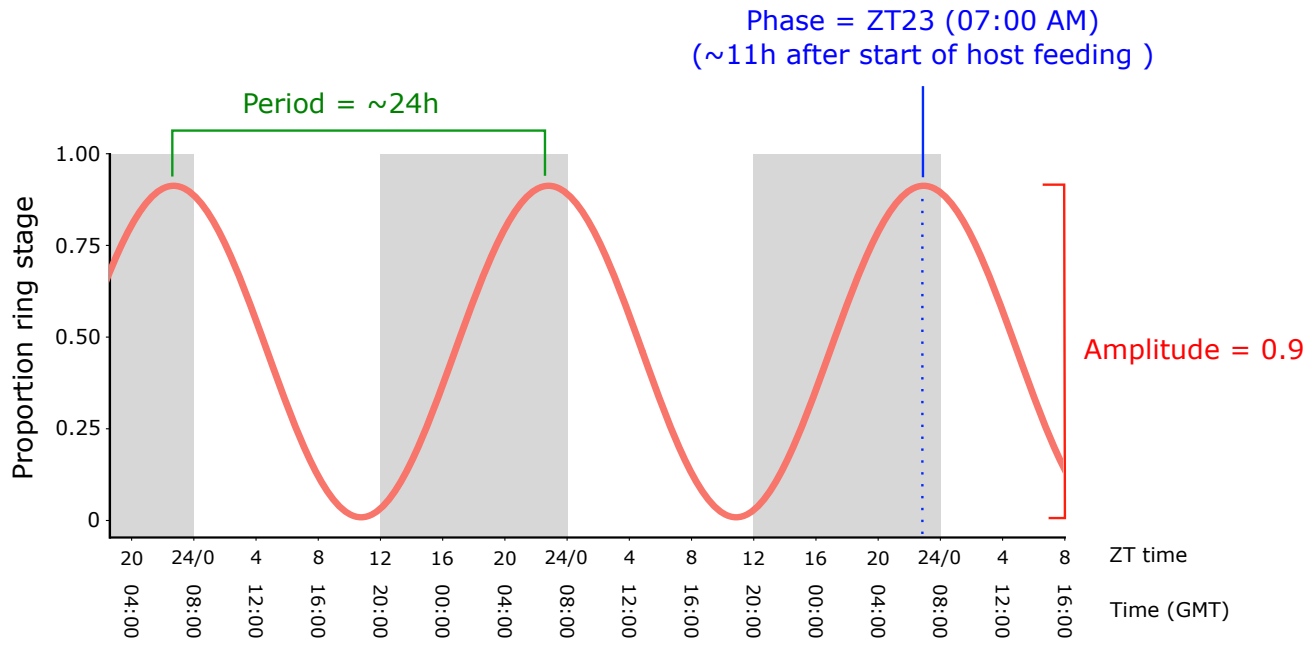

Figure 7: An illustrative example of the rhythm parameters for a synchronous infection. Shown are the proportion of parasites in the ring stage. Ring stages exhibit high amplitude rhythms with a peak at the end of the dark period/feeding period and typically reoccur with a 24 hour frequency. Shading represents lights off (i.e. the photo-schedule) and is also analogous to the host's feeding window. Time is given in Zeitgeber time (hours relative to lights ON) and in GMT.

How?

When the host's rhythm is perturbed relative to the phase of the IDC, the IDC schedule readily alters to regain alignment with host rhythms and resynchronizes if IDC synchrony is perturbed. As detailed below, rescheduling and synchronization occur via a faster IDC which advances the peak phase of IDC stages Figure 8)(*paper #6)*.

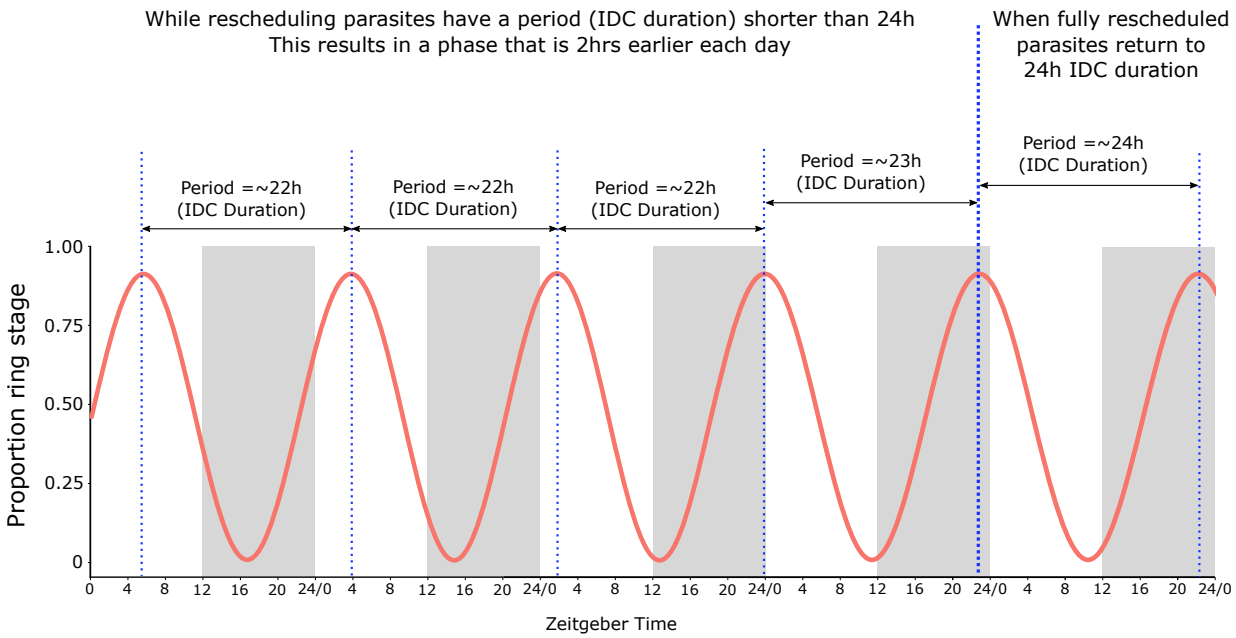

Figure 8: The relationship between IDC period and rescheduling of a mismatched infection. Parasites begin out of alignment with the host, with a peak in ring stage parasites (IDC phase; blue dotted lines) occurring during the host's light / fasting period. An IDC period shorter than 24hrs results in an earlier phase each day. Within 5 cycles the parasite has realigned with the host and the IDC phase occurs at the end of the host's dark / feeding period. Once realigned the IDC period returns to ~24h to maintain the schedule. Shaded areas indicate lights OFF (i.e. the host's photo-schedule) and is also analogous to the host's feeding window.

Phase (Timing)

Following perturbation of the temporal alignment between host and parasite rhythms, the phase of the IDC schedule changes very little for the first 2-3 cycles. Yet, parasites mismatched to the host's rhythm, even by 12 hours, become fully rescheduled within five (*paper #6*) to seven (*papers #1-2*) cycles. More in depth investigation in *paper #6*, revealed that phase – defined as when the ring stage peaks – advances by 2-3 hours per day until the IDC becomes aligned with host rhythms in manner illustrated in Figure 8. Thus, parsimony suggests the phase of all IDC stages advances in concert with the ring stage. These phase changes cannot be explained simply be selective death of a mistimed IDC stage at a particular time-of-day, or by sequestration obscuring an accurate estimation of the stage distribution of the IDC during certain sampling times (*papers #1 and #6*). Furthermore, the rate at which the IDC reschedules is independent of parasite density (i.e. perturbation of the dose of parasites used to initiate infections) (*paper #6*).

The driver for rescheduling is the host's feeding-fasting rhythm (Hirako et al. 2018, Prior et al. 2018). O'Donnell 2020 (*paper #4*) confirmed that the ring stage peak occurs at the end of the host's feeding period. A role for light-dark schedules was excluded based on the observations that the IDC schedule aligns with the timing of feeding/fasting in day fed and night fed mice, regardless of the phase of light entrained (SCN) clocks, and when free-running in constant darkness (DD). Further investigation in *paper #4* ruled out involvement of host TTFL clocks in the IDC schedule. Specifically, the IDC schedule becomes inverted in clock impaired mice with a feeding-fasting rhythm if infections are initiated with ring stages before the start of the feeding window (because the ring stage shifts to its normal peak at the end of the feeding window). Yet, the IDC rhythm becomes dampened in clock impaired mice without a feeding-fasting rhythm (*ad lib* feeding). Analogous results occur when infections are initiated with an asynchronous mix of IDC stages. In clock impaired mice, the IDC becomes synchronised and ring stages peak at the end of the feeding window when hosts experience TRF, but the IDC remains asynchronous in hosts without a feeding-fasting rhythm. Thus, a feeding-fasting rhythm alone is sufficient to set the phase of the IDC rhythm and a host TTFL is not essential.

The phase of the IDC rhythm exhibits little within-treatment variation during the first 6 days of infection, even in rescheduling treatment groups in which the phase is changing each day. Conversely, during the post-peak window, when hosts are sick, parasite phase is quite variable within treatments (*paper #6*). This post-peak increase in variation is observed across all treatment groups so is unlikely to be due to rescheduling or being in an arrhythmic host and may be linked to host sickness.

Synchrony (Amplitude)

The experiments described above demonstrate that a feeding-fasting rhythm synchronizes the IDC, and in the absence of such a rhythm, the IDC becomes dampened or remains asynchronous (*paper #4*). Infections initiated with synchronous parasites remained highly synchronised (amplitudes of 0.9-0.96), in rhythmic hosts despite rescheduling in some cases, but became dampened (amplitude of ~0.4) in arrhythmic hosts.

When parasites are forced to synchronise, as well as establish the correct phase relationship to the host feeding-fasting rhythm, greater synchrony is achieved in clock impaired hosts with a feeding-fasting rhythm (0.85) than in control infections of wild type mice (0.75). Furthermore, when the host's light-entrained rhythms are decoupled from the phase of feeding-fasting rhythms, the IDC rhythm had an intermediate level of synchrony (~0.6).

To assess whether synchrony changes during the process of rescheduling, *paper #6* observed parasite synchrony throughout days 2-10 post infection. Synchrony remains stable for the first 6 days of the infection (the pre-peak infection window) but following the peak in asexual density, synchrony degrades when hosts become very symptomatic. Like parasite phase, the post-peak loss of synchrony is universal across infections (regardless of the perturbations applied) and thus, is not correlated with rescheduling or with being inside an arrhythmic host, and is likely to be simply due a deterioration in host health. In addition, when arrhythmic hosts are group housed (*paper #6*), the IDC rhythm does not dampen as much as when arrhythmic hosts are singly housed (*paper #4*).

Given the strong and repeatable influence of host rhythms on *P. chabaudi,* O'Donnell 2019 (*paper #5*) tested whether perturbations to host rhythms can generate synchrony in the asynchronous species, *P.*

berghei. In contrast to suggestions from historical literature (Arnold et al. 1969), a long day photoperiod does not synchronise *P. berghei*. Further, applying a feeding-fasting regime to clock impaired mice has no impact on *P. berghei*, which shows similarly arrhythmic IDC stage proportions to infections in clock impaired mice with constant access to food.

Period (IDC duration)

Having observed that the IDC can readily become rescheduled without a loss of synchrony, O'Donnell 2021b (*paper #6*) asked whether parasites reschedule simply by altering the period of the IDC. For example by pausing for 12 hours to recover from a 12 hour mismatch to the host's rhythm, or by speeding up / slowing down each cycle to gradually reach the correct phase alignment. Compared to control infections with a period of 23.4 hours, mismatched parasites exhibit periods 1-2 hours shorter during rescheduling (periods of around 21.3-22.5 hours). Infections in clock impaired hosts without feeding rhythms were also short (22.5h), potentially suggesting a short free-running period. Finally, as observed for phase and synchrony, during the post peak window of the infections period estimates are more uncertain across all infections (regardless of the perturbations applied), reflective of the disruption caused when hosts become symptomatic.

Why?

In addition to increasing understanding of the proximate drivers of the IDC rhythm, my portfolio also probed why parasites should schedule their IDC.

Within-host survival

My portfolio begins with a publication in 2011 (*paper #1*) that resurrects the topic of explaining why IDC rhythms occur, following a flurry of activity several decades ago. This paper took a different approach to the puzzle by asking whether the IDC rhythm is a fitness-related trait. Following a 12 hour mismatch to host rhythms, twice daily measurements revealed a 50% reduction in asexual and sexual parasite densities across the pre-peak window compared to control infections that were initiated in alignment to host rhythms (*paper #1*). Given that asexual replication underpins within-host survival and gametocyte density reflects transmission potential, the timing of the IDC schedule appears to have significant fitness consequences.

Further investigation focused on asexual densities, testing whether the costs of mismatch to host rhythms depended on whether the route of entry into the host (directly into the blood or via the peritoneal cavity) and the IDC stage (rings or late trophozoites) used to initiate infections (*paper #2*). Higher densities always occur in infections started intravenously, but the route of infection did not influence the impact of misalignment to host rhythms. Infections initiated with ring stages suffered a 40% reduction in cumulative asexual density, supporting the findings in *paper #1*. However, in contrast to expectation, infections initiated with trophozoites benefited from misalignment to host rhythms, achieving a cumulative density 60% higher than counterparts matched to host rhythms. The different impacts on ring and trophozoite stages also correlate with the time-of-day infections were initiated. For example, infections must occur in the morning for rings to be matched to host rhythms and for trophozoites to be mismatched; and these are the infections that perform best.

When misalignment to host rhythms has negative consequences for asexual replication, its impact (a 35% reduction in parasite density relative to matched infections) is detectable on the 2nd day following infection (*paper #2*). Because asexual replication is exponential, any differences in survival or replicative potential become magnified as infections progress. However, more recent experiments, which use a different parasite genotype to force parasites to reschedule do not find an overt cost of mismatch (*paper #6*). Specifically, parasites that are mismatched to the phase of feeding-fasting rhythms in wild type hosts whose feeding-fasting rhythms are (i) aligned or (ii) not with light-entrained rhythms, or (iii) in clock impaired mice (which only have a feeding fasting rhythm), perform just as well as parasites that begin infections in alignment with feeding-fasting rhythms in all these cases (*paper #6*). Yet, a hidden cost is present; because rescheduling parasites have a faster IDC, their densities should exceed those of control parasites if there were no costs of speeding up the IDC. The consequences of infecting a host with a disrupted TTFL are less clear; parasites sometimes perform less well in clock impaired mice than wild types (*paper #4*) but not consistently (*paper #6*). In keeping with the lack of impact of host feeding-fasting rhythms on *P. berghei*'s IDC schedule, perturbations to host rhythms do not affect its asexual density dynamics either (*paper #5*).

Between-host transmission

In addition to host and parasite rhythms affecting the density of sexual transmission stages (*paper #1*), rhythms can affect other aspects of transmission (Rund et al. 2016, Schneider et al. 2018). The rhythms of all three parties - hosts, parasites and mosquitoes – involved could influence transmission directly as well as via synergistic or antagonistic effects of interactions between their rhythms. Reports of vectors altering their blood seeking rhythms to evade insecticide treated bed nets requires the role of vector time-of-day for transmission to be urgently investigated. *Paper #3* addresses this issue by testing whether blood feeding during the day instead of during the evening affects parasite transmission and mosquito fitness (using proxies relating to survival and reproduction). Note, the asynchronous species *P. berghei* was used to allow the impacts of host and parasite timing to be tested independently of parasite rhythms.

Time-of-day of the blood meal had no impact on parasite transmission (proportion of mosquitoes infected) or on parasite burden (number of oocysts per midgut). This negative result is supported by the lack of variation in gametocyte densities in the host blood in the evening versus the morning. As expected, infection reduced mosquito lifespan (by ~3 days) and the daily hazard of dying for infected mosquitoes was ~50% higher than for uninfected mosquitoes, but survival was not influenced by time-of-day of blood feeding.

Mosquito lifespan was not affected by the change in blood meal timing, nor by host time-of-day or the quality (anaemic or healthy) of blood. A change in blood meal timing had only minor effects on mosquito reproduction. Mosquitoes that fed in the morning were more likely to lay eggs (17% more morning fed females than evening fed females laid eggs), laid on average half a day earlier than night fed mosquitoes, and had ~7% larger clutch sizes. Independent of the time-of-day effects, blood quality influenced the volume of the blood meal taken with mosquitoes feeding on anaemic hosts taking up ~25% larger blood meals than those feeding on control hosts and blood quality also had consequences for clutch size with 12-35% larger clutch sizes for individuals that fed on control versus anaemic hosts.

The other paper in my portfolio concerning transmission investigates whether *P. berghei* sexual stages exhibit rhythms in their activities (*paper #5*). Both the densities of female gametocytes in the blood and

the rate of male gametogenesis (exflagellation; the act of replication and formation of flagellated microgametes unique to male gametocytes which occurs in the mosquito midgut) were both statistically rhythmic with a peak density/activity in the evening.

Conclusions

Parasites are an important part of ecosystems and daily rhythms in environmental conditions due to the rotation of the Earth shape ecological interactions between hosts and parasites. Mounting evidence reveals that diverse parasites have evolved a myriad of traits to take advantage of the opportunities that daily rhythms bring, or cope with the constraints they impose (Reece et al. 2017, Rijo-Ferreira et al. 2017, Prior et al. 2020). However, the nature of parasite rhythms and the extent to which they result from activities encoded by the genes of hosts and parasites are poorly understood. Further, in cases where parasites can organize their own schedule (either via an endogenous oscillator or a simpler time keeping strategy), why these abilities have evolved (i.e. their selective drivers) are also largely unknown. The work presented my portfolio addresses these issues, providing a detailed description of rhythmicity in cycles of asexual replication undertaken by malaria parasites in the blood of their vertebrate host, revealing new insight into how this rhythm is established, and exploring whether parasites garner fitness benefits in terms of survival and transmission.

What are the characteristics of the IDC rhythm?

I have applied approaches from chronobiology that include well established experimental paradigms and circular statistics, to bring a quantitative framework to studying 'periodicity' in malaria parasites, which has been a long standing topic of interest. Across my experiments, the IDC schedule is very repeatable and is characterized by a high amplitude (~0.9), 24h rhythm, that has a peak phase around ZT23 (11 hours after the start of host feeding) during the pre-peak phase of the infection and becomes dampened and disrupted post-peak, with lower rhythm amplitude (<0.5) and variable phase.

By improving resolution on the features of the IDC rhythm, my work paves the way for others to begin identifying the genetic and molecular mechanisms involved. By splitting the IDC rhythm into its quantitative traits (IDC timing, IDC synchrony and IDC duration) it becomes possible to ask questions about the extent to which they are independent targets of selection or constrained by pleiotropy. This can be done independently of understanding the mechanism by which parasites control the IDC schedule and can inform the search for mechanism. For example, future work may demonstrate genetic variation (i.e. differences between genotypes) in some rhythmicity parameters suggesting that the genes underpinning this variation are exposed to selection. For example, rhythm parameters may span greater variation across genotypes in some within-host conditions (such as sickness) than others (such as health). Under this scenario, any perturbations leading to sicker hosts will increase the amount of parasite genetic variation exposed to selection. Further, if some parameters are more variable between genotypes than others (especially if their relationships qualitatively differ across genotypes), then they may be encoded by different genes. Harnessing parasite responses to different perturbations of host rhythms and variation across parasite genotypes could be provide useful context for those searching for signatures of time-keeping mechanisms in transcriptomic data sets (Rijo-Ferreira et al. 2020).

How is the IDC rhythm established?

Host feeding-fasting rhythms are normally entrained by photo-schedule, such that nocturnal mice feed at night, so IDC completion occurs towards the end of the dark phase. However, my portfolio and the work of others demonstrates that photo-schedule plays no direct role in the IDC schedule. Body temperature is another rhythm that usually light entrained, and so, in phase with activity rhythms. For small animals, such as mice that have low thermal inertia, metabolism following feeding also has a direct effect on body temperature (Sojka et al. 2013). Whilst temperature is well known to affect the duration of the IDC of parasites in culture, my experiments (*particularly paper #4*) indirectly suggest it is unlikely to be time-of-day cue that parasites use *in vivo*. My work also conclusively demonstrates that canonical host TTFL clocks do not have an essential role in the IDC schedule, and these rhythms may slightly erode the parasites ability to align specifically to feeding-fasting rhythms.

If parasites actively schedule the IDC themselves, the timing cue/Zeitgeber they use should be reliable (i.e. only transiently available at same time each day), and so, not something the parasites can generate or scavenge at any time-of-day. Several candidate timing cues have been proposed including melatonin, glucose and cytokines (Bagnaresi et al. 2012, Hirako et al. 2018, Prior et al. 2018). Melatonin appears to advance IDC completion *in vivo* but the concentrations needed to elicit this effect were 10 fold higher than found in the blood of humans or mice. Glucose was also proposed as a cue given the observation that parasite stages differ in their glucose requirements e.g. schizonts, at the end of the IDC, consume large amounts of glucose (Olszewski et al. 2011, Hirako et al. 2018). Further, the increase in immune cell activity as a result of infection also consumes glucose making the daily glucose rhythms theoretically more extreme under infection (Hirako et al. 2018). However, glucose rhythms are very damp and not always in phase with the IDC schedule across different perturbations of host TTFL and feeding regimes (Prior et al. 2018, Prior et al. 2021). A recent metabolomics screen of parasites in mismatched and matched infections reveals the most promising candidate cue, isoleucine (Prior et al. 2021). This amino acid is one of the few amino acids that exhibits a daily cycle in mice and humans and peaks at the end of the feeding period. As the parasite is unable to synthesis isoleucine itself, and it is minimally present in haemoglobin, isoleucine is entirely scavenged from host blood. When deprived of isoleucine in culture, both human and rodent malaria parasites dramatically slow cell cycle progression akin to dormancy, yet are able to recover upon addition of isoleucine (Babbitt et al. 2012, McLean et al. 2020, Prior et al. 2021). It's possible that parasites have more than one cue, however, testing this *in vitro* is difficult because isoleucine is also essential to the host and cannot be removed.

That parasites starting their infections mismatched to host feeding rhythms by 12 hours reschedule in around 5 IDC cycles is another repeatable result from my portfolio (*papers #1, #2, #4, and #6*) and others (Prior et al. 2018, Subudhi et al. 2020). Rescheduling parasites speed up their IDC by ~2 hours per cycle, allowing them to gradually change phase each day. The mechanism by which parasites control IDC duration is unknown but the gene Serpentine Receptor 10 (SR10), may be involved (Subudhi et al. 2020). SR10 cycles with a duration close to 24 hours in *P. chabaudi* and *P. falciparum* (which has a 48h IDC duration) and knocking out this gene in *P. chabaudi* resulted in IDC durations 2-3 shorter than normal along with several 'SR10-linked' genes losing rhythmicity (Subudhi et al. 2020). Perhaps parasites only express SR10 when in alignment with host rhythms? Further, this approach to rescheduling rules out the suggestion that misaligned parasites might become dormant (as they do in response to the stress of drug treatment) until they detect they are at the correct time-of-day for the IDC stage they are at. Or, that selective host killing of misaligned stages IDC stages enforces a schedule upon parasites. The

current most likely scenario is that parasites possess a time-keeping ability to ensure they align with the resources they require from the host's food, but that – at least when at low densities and in well fed lab mice – mass death is not a consequence of temporary misalignment. If, for example, isoleucine is the selective driver for the phase of the IDC, then using it as a time cue/Zeitgeber ensures parasites respond to the most accurate (i.e. relevant) timing information. However, a down-side of this strategy may be that parasites do not have an opportunity to anticipate and prepare, which responding to a proxy that occurs in advance of isoleucine could offer them.

Why have parasites evolved to schedule the IDC?

That parasites have evolved plasticity in the IDC schedule suggests that being matched to the host's rhythms is of considerable benefit. Studying the fitness consequences of misaligned parasite rhythms can offer insight into why they are beneficial. Early papers in my portfolio find tangible fitness benefits in terms of asexual densities and gametocyte densities which underpin within-host survival and between-host transmission, respectively.

Subsequent research found that the reduction in gametocyte density is not due to decreased investment in gametocytes (which parasites often do when experiencing a reduction in asexual replication (Reece et al. 2008, Reece et al. 2010, Cameron et al. 2013)), nor is it explained by misaligned gametocytes being at a vulnerable stage and experiencing a higher mortality risk when the inflammatory cytokine spike associated with schizogony occurs (Westwood et al. 2020). Instead of misalignment to host rhythms causing the loss of gametocytes, recent work suggests that a daily rhythm in gametocyte density is to be expected based on their short lifespan (Schneider et al. 2018). Specifically, in normal circumstances, gametocytes are produced the night, reach sexual maturity the following night and then senesce and are cleared over the subsequent 24 hours. This means that at night, the gametocyte population is composed mostly of newly matured gametocytes because those from the previous cohort have been cleared, but earlier in the day the previous, senescing cohort are still present. Thus, depending on how the timing of gametocyte maturation and senescence proceeds in misaligned infections, coupled with the timing of sampling, misaligned infection may appear to have fewer gametocytes but actually, they have fewer senesced gametocytes. Studies that track gametocyte density and infectivity from intensive sampling regimes (as I have done for the IDC) may resolve how misalignment affects the transmission component of parasite fitness. Because a rhythmic IDC imposes rhythms in gametocyte production, parasites might have been selected to fine-tune gametocyte maturation to ensure optimal infectivity when vectors forage for blood. Alternatively, gametocyte development might be constrained and the timing of the IDC selected to ensure gametocyte production occurs on time, and there are coincident benefits of aligning with rhythmicity in the resources required from host food.

Understanding the consequences of misalignment to host rhythms for asexual stages has also proved complex. It appears that the main impact occurs in the first 48 hours of the infection and only when mismatched infections are started with ring stage parasites (i.e. rings going into evening hosts). This may be explained by host rhythms because many murine immune factors are upregulated during the active phase (in the evening) thus posing a higher risk for new infections (Keller et al. 2009, Scheiermann et al. 2013, O'Donnell et al. 2014). Additionally, if "foreign" donor RBCs are an additional target for clearance by the immune system, later stages (i.e. trophozoites) are exposed to these responses for a shorter window before they reach schizogony and are then be able to reinvade the host's own RBCs. These

explanations could be tested by comparing the dynamics of infections initiated by rings or trophozoites in arrhythmic (clock mutant) mice. Also unexplained is why an overt cost of misalignment was not evident in my more recent experiments. These experiments used a different genotypes for host and parasite, so the costs may depend on parasite virulence, with more virulent genotypes being more vulnerable (perhaps because they need more resources to fuel their higher replication rate). However, misaligned parasites complete one more IDC than control parasites during rescheduling, yet do not reach higher density, which is likely to reflect some costs of misalignment. The number of progeny per parasite is a plastic trait altered in response to changes in within host conditions and so, parasites might not be to produce as many progeny in a shorter IDC. Furthermore, rescheduling is associated with disruption of transcriptional patterns related to important cellular processes (Subudhi et al. 2020).

I also find that parasite synchrony degrades when infections reach peak parasitemia and that this occurs regardless of whether parasites began infections aligned to host rhythms or had to reschedule, or whether hosts were wild type or clock disrupted mutants. Notably, parasites in infections aligned to host rhythms experienced the least alteration to the IDC schedule, suggesting a well-established rhythm is less vulnerable to perturbation. Host rhythms become perturbed at the peak of infection, they lose weight and become anorexic as well as the parasites experiencing red blood cell limitation due to anaemia. There are no obvious consequences of the degree of IDC rhythm disruption for within-host survival but due to increased variation between (replicate) infections within the same treatment group, higher powered experiments are necessary to test this.

Whilst *P. chabaudi's* default is a rhythmic IDC, I found (*paper #5*) that species such as *P. berghei* always exhibit an arrhythmic IDC, without any observable costs to fitness. The experimental manipulations that generate synchrony in *P. chabaudi* have no effect on *P. berghei* suggesting that this parasite lacks the machinery to establish and maintain rhythms; either a yet to be defined internal oscillator, or simply the ability to detect and respond to transient resources. Alternatively, arrhythmicity and synchrony, sitting at opposite ends of the spectrum may be equally good strategies in a rhythmic host (Greischar et al. 2014). For example, arrhythmic replication might minimize competition between related parasites for resources. However, given that *P. berghei* does not reach the asexual densities of *P. chabaudi*, avoiding such 'scramble' competition is unlikely to be necessary. Instead, perhaps due to its low densities, *P. berghei* can acquire sufficient resources around the clock and so, would not benefit from paying the costs associated with maintaining mechanisms required to be rhythmic. Additionally, rodent may not be the only natural host for *P. berghei* so arrhythmicity could be a product of living in an unusual environment. Infection dynamics of *Plasmodium* infections in thicket rats do differ from lab mice with infections reaching higher parasitemaia and taking longer to get to peak (Conteh et al. 2020), but there is no information on the IDC schedule exhibited in this host. Bats may also be a natural host for *P. berghei* as other *Plasmodium* species that infect bats are closely related to *P. berghei* and the mosquito vector of *P. berghei* likely also bite bats (Schaer et al. 2013). Both rodents and bats have similar nocturnal feeding patterns but their diets vary. The natural rat host for *P. berghei* (Grammomys surdaster) is a generalist and feeds on a variety of plants and insects while Congolese bats are exclusively frugivorous or insectivorous (Schaer et al. 2013). Perhaps such an exclusivity of diet types leads to host-dependent differences in the timing and duration of digestion which the parasites are unable to predict. Arrhythmicity may be a catch-all strategy that allows *P. berghei* to cope with any host differences in the timing of available resources.

Despite no evidence of IDC rhythmicity in *P. berghei*, I did detect weak evidence of daily rhythms in female gametocyte density and in male gametocyte activity, which are mostly likely driven by rhythms in host immune factors that can interfere with transmission. However, neither time-of-day for parasites, or for mosquito vectors, impact on *P. berghei*'s transmission success. There are increasing reports that to evade insecticide treated bed nets, mosquitoes are altering the timing of their flight and biting activity to forage for blood that is earlier in the day (Yohannes et al. 2012, Sougoufara et al. 2014, Cooke et al. 2015, Wamae et al. 2015). Given that many mosquito rhythms are light-entrained, disrupting the phase relationship between feeding and other rhythms may be detrimental to mosquitoes (analogous to shift work in humans). However, I found only minor consequences of day-biting for mosquito reproduction and no effect on mosquito lifespan. Thus, it appears unlikely that advancing mosquito biting time will have an additional impact on transmission dynamics (other than allowing transmission to occur as it did before bed nets). However, future work should consider less extreme changes in biting timing and consider mosquitoes that experience more ecologically relevant stressors than those I well-resourced lab colonies.

From mice to humans

Can insights gained from studying a rodent malaria model system in which the IDC duration is around 24h be beneficial for a human malaria system where the IDC durations are either 24, 48 or 72 hours (depending on species)? And can rhythms relevant to living in a nocturnal host simply be inverted for parasites living in humans? Humans and mice share 24h rhythms in immune activity (Scheiermann et al. 2013) and in the release of RBCs from the bone marrow (Clark et al. 1969, McKee et al. 1974), the phase of which is relative to each host's active period. However, despite the relevance of these host processes to parasites, our work suggests that parasites primarily align to host feeding rhythms and mice and humans may have similar or different feeding windows depending on which human meal is the largest. Mice take a large meal at the start of their active phase (ZT13) while the timing of the largest meal for humans can occur 8 hours earlier (lunch; ZT5) or at a similar time-of-day to mice (dinner; ZT12). For a 24 hour human malaria parasites (*P. knowlesi*), any differences in feeding rhythms could simply be reflected in a different parasite phase, in the same way *P. chabaudi* changes phase in light fed TRF mice, rescheduling so the energy demanding parasite stages align with the transient food resource. It is unclear why the IDC is synchronous in a parasite such as *P. falciparum* that spends 24 of their 48h development in a "feeding" parasite stage, thus guaranteeing that they are always present for the window of opportunity irrespective of host phase. Unlike *P. chabaudi* (Subudhi et al. 2020) very few *P. falciparum* genes cycle with a periodicity close to 24hours (Smith et al. 2020) suggesting that daily host rhythms are simply not as important these parasites (however time-series transcriptomics has only been carried out on parasites that are adapted to culture where there is little selective advantage to being in phase with environmental rhythms). Another key difference between the lab infections in mice used in my experiments and natural human infections is that human infections are often composed of multiple parasites genotypes. How parasite rhythms change in response to competition is still unknown but with the multiple rodent malaria parasite genotypes available this could be tested more effectively in mice than in humans (particularly compared to *P. falciparum in vitro*).

Understanding how and why human parasites generate and maintain rhythms could inform treatment in several ways: (i) interfering with the mechanics of the IDC schedule may help overcome the problem of parasites invoking dormancy to tolerant drug treatment. (ii) knowledge of the molecular mechanism of parasite time keeping may inform targets for new drugs (e.g. drugs that detrimentally disrupt IDC

duration could reduce disease severity and transmission potential), (iii) drugs could be more effective if administered at particular times of day to specifically kill the most vulnerable IDC stages, or to synergise with host rhythms. (iv) some vaccines have time-of-day efficacy (Barnoud et al. 2021), and improving on the low rate of protection provided by the first malaria vaccine to be deployed would be of great benefit. More generally, understanding malaria rhythms facilitates better predictions of the consequences of a changing environment e.g. the range expansion of vectors with different behavioural timing, or the changing of local vector biting time in response to control measures (Thomsen et al. 2017).

References

Arnold, J. D., F. Lalli and D. C. Martin (1969). "Augmentation of growth and division synchrony and of vascular sequestration of *Plasmodium berghei* by the photoperiodic rhythm." The Journal of Parasitology **55**(3): 597-608.

Babbitt, S. E., L. Altenhofen, S. A. Cobbold, E. S. Istvan, C. Fennell, C. Doerig, M. Llinás and D. E. Goldberg (2012). "*Plasmodium falciparum* responds to amino acid starvation by entering into a hibernatory state." Proceedings of the National Academy of Sciences **109**(47): E3278-E3287.

Bae, K., X. Jin, E. S. Maywood, M. H. Hastings, S. M. Reppert and D. R. Weaver (2001). "Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock." Neuron **30**(2): 525-536.

Bagnaresi, P., M. Nakabashi, A. P. Thomas, R. J. Reiter and C. R. Garcia (2012). "The role of melatonin in parasite biology." Molecular & Biochemical Parasitology **181**(1): 1-6.

Baraldo, M. (2008). "The influence of circadian rhythms on the kinetics of drugs in humans." Expert opinion on drug metabolism & toxicology **4**(2): 175-192.

Barnoud, C., C. Wang and C. Scheiermann (2021). "Timing vaccination against SARS-CoV-2." Cell Research: 1-2.

Baxter, M. and D. W. Ray (2020). "Circadian rhythms in innate immunity and stress responses." Immunology **161**(4): 261-267.

Beaver, L., B. Gvakharia, T. Vollintine, D. Hege, R. Stanewsky and J. Giebultowicz (2002). "Loss of circadian clock function decreases reproductive fitness in males of Drosophila melanogaster." Proceedings of the National Academy of Sciences **99**(4): 2134-2139.

Bellet, M. M., E. Deriu, J. Z. Liu, B. Grimaldi, C. Blaschitz, M. Zeller, R. A. Edwards, S. Sahar, S. Dandekar and P. Baldi (2013). "Circadian clock regulates the host response to Salmonella." Proceedings of the National Academy of Sciences **110**(24): 9897-9902.

Bhardwaj, V., S. Meier, L. N. Petersen, R. A. Ingle and L. C. Roden (2011). "Defence responses of Arabidopsis thaliana to infection by Pseudomonas syringae are regulated by the circadian clock." PloS one **6**(10): e26968.

Birget, P. L., K. F. Prior, N. J. Savill, L. Steer and S. E. Reece (2019). "Plasticity and genetic variation in traits underpinning asexual replication of the rodent malaria parasite, Plasmodium chabaudi." Malaria journal **18**(1): 1-11.

Boyd, G. (1929). "Experimental modification of the reproductive activity of Plasmodium cathemerium." Journal of Experimental Zoology **54**: 111 - 126.

Bray, R. S., A. W. R. McCrae and M. E. Smalley (1976). "Lack of a circadian rhythm in the ability of the gametocytes of *Plasmodium falciparum* to infect *Anopheles gambiae*." International journal for parasitology **6**(5): 399-401.
Cambie, G., V. Caillard, A. Beaute-Lafitte, H. Ginsburg, A. Chabaud and I. Landau (1991). "Chronotherapy of malaria: identification of drug-sensitive stage of parasite and timing of drug delivery for improved therapy." Annales de parasitologie humaine et comparée **66**(1): 14-21.
Cameron, A., S. E. Reece, D. R. Drew, D. T. Haydon and A. J. Yates (2013). "Plasticity in transmission strategies of the malaria parasite, *Plasmodium chabaudi*: environmental and genetic effects." Evolutionary applications **6**(2): 365-376.
Carter, L. M., B. F. Kafsack, M. Llinás, N. Mideo, L. C. Pollitt and S. E. Reece (2013). "Stress and sex in malaria parasites: Why does commitment vary?" Evolution, medicine, and public health **2013**(1): 135-147.
Cavallari, N., E. Frigato, D. Vallone, N. Fröhlich, J. F. Lopez-Olmeda, A. Foa, R. Berti, F. J. Sánchez-Vázquez, C. Bertolucci and N. S. Foulkes (2011). "A blind circadian clock in cavefish reveals that opsins mediate peripheral clock photoreception." PLoS biology **9**(9): e1001142.
Chen, Z., E. A. Odstrcil, B. P. Tu and S. L. McKnight (2007). "Restriction of DNA replication to the reductive phase of the metabolic cycle protects genome integrity." Science **316**(5833): 1916-1919.
Clark, R. H. and D. R. Korst (1969). "Circadian periodicity of bone marrow mitotic activity and reticulocyte counts in rats and mice." Science **166**(3902): 236-237.
Conteh, S., J. Kolasny, Y. L. Robbins, P. Pyana, P. Büscher, J. Musgrove, B. Butler, L. Lambert, J. P. Gorres and P. E. Duffy (2020). "Dynamics and outcomes of *Plasmodium* infections in *Grammomys surdaster* (*Grammomys dolichurus*) thicket rats versus inbred mice." The American Journal of Tropical Medicine and Hygiene **103**(5): 1893-1901.
Cooke, M. K., S. C. Kahindi, R. M. Oriango, C. Owaga, E. Ayoma, D. Mabuka, D. Nyangau, L. Abel, E. Atieno, S. Awuor, C. Drakeley, J. Cox and J. Stevenson (2015). "'A bite before bed': exposure to malaria vectors outside the times of net use in the highlands of western Kenya." Malaria Journal **14**.
DeCoursey, P., J. Walker and S. Smith (2000). "A circadian pacemaker in free-living chipmunks: essential for survival?" Journal of Comparative Physiology A **186**(2): 169-180.
DeCoursey, P. J. and J. R. Krulas (1998). "Behavior of SCN-lesioned chipmunks in natural habitat: a pilot study." Journal of biological rhythms **13**(3): 229-244.
Deharo, E., F. Coquelin, A. G. Chabaud and I. Landau (1996). "The erythrocytic schizogony of two synchronized strains of *Plasmodium berghei*, NK65 and ANKA, in normocytes and reticulocytes." Parasitology research **82**(2): 178-182.
Dimitrov, S., C. Benedict, D. Heutling, J. Westermann, J. Born and T. Lange (2009). "Cortisol and epinephrine control opposing circadian rhythms in T cell subsets." Blood, the Journal of the American Society of Hematology **113**(21): 5134-5143.
Dodd, A. N., N. Salathia, A. Hall, E. Kevei, R. Toth, F. Nagy, J. M. Hibberd, A. J. Millar and A. A. R. Webb (2005). "Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage." Science **309**(5734): 630-633.
Doi, M. (2012). "Circadian clock-deficient mice as a tool for exploring disease etiology." Biological and Pharmaceutical Bulletin **35**(9): 1385-1391.
Dowse, H. B. (2013). "Maximum entropy spectral analysis for circadian rhythms: theory, history and practice." Journal of circadian rhythms **11**(1): 1-9.
Dumbell, R., O. Matveeva and H. Oster (2016). "Circadian clocks, stress, and immunity." Frontiers in endocrinology **7**: 37.
Dunlap, J. C. (1999). "Molecular bases for circadian clocks." Cell **96**(2): 271-290.

Edgar, R. S., E. W. Green, Y. Zhao, G. Van Ooijen, M. Olmedo, X. Qin, Y. Xu, M. Pan, U. K. Valekunja and K. A. Feeney (2012). "Peroxiredoxins are conserved markers of circadian rhythms." Nature **485**(7399): 459-464.
Edgar, R. S., A. Stangherlin, A. D. Nagy, M. P. Nicoll, S. Efstathiou, J. S. O'Neill and A. B. Reddy (2016). "Cell autonomous regulation of herpes and influenza virus infection by the circadian clock." Proceedings of the National Academy of Sciences **113**(36): 10085-10090.
Emerson, K. J., W. E. Bradshaw and C. M. Holzapfel (2008). "Concordance of the circadian clock with the environment is necessary to maximize fitness in natural populations." Evolution: International Journal of Organic Evolution **62**(4): 979-983.
Faltýnková, A., A. Karvonen, M. Jyrkkä and E. Valtonen (2009). "Being successful in the world of narrow opportunities: transmission patterns of the trematode Ichthyocotylurus pileatus." Parasitology **136**(11): 1375-1382.
Feeney, K. A., L. L. Hansen, M. Putker, C. Olivares-Yañez, J. Day, L. J. Eades, L. F. Larrondo, N. P. Hoyle, J. S. O'Neill and G. van Ooijen (2016). "Daily magnesium fluxes regulate cellular timekeeping and energy balance." Nature **532**(7599): 375-379.
Franke-Fayard, B., J. Fonager, A. Braks, S. M. Khan and C. J. Janse (2010). "Sequestration and tissue accumulation of human malaria parasites: can we learn anything from rodent models of malaria?" PLoS pathogens **6**(9): e1001032.
Garcia, C. R., R. P. Markus and L. Madeira (2001). "Tertian and quartan fevers: temporal regulation in malarial infection." Journal of Biological Rhythms **16**(5): 436-443.
Garnham, P. C. C. and K. G. Powers (1974). "Periodicity of infectivity of plasmodial gametocytes: The Hawking Phenomenom." International journal for parasitology **4**(1): 103-106.
Gautret, P., E. Deharo, R. Tahar, A. G. Chabaud and I. Landau (1995). "The adjustment of the schizogonic cycle of *Plasmodium chabaudi chabaudi* in the blood to the circadian rhythm of the host." Parasite **2**(1): 69-74.
Gautret, P., F. Miltgen, J. C. Gantier, A. G. Chabaud and I. Landau (1996). "Enhanced gametocyte formation by *Plasmodium chabaudi* in immature erythrocytes: Pattern of production, sequestration, and infectivity to mosquitoes." Journal of Parasitology **82**(6): 900-906.
Gazzinelli, R. T., P. Kalantari, K. A. Fitzgerald and D. T. Golenbock (2014). "Innate sensing of malaria parasites." Nat Rev Immunol **14**(11): 744-757.
Githeko, A. K., A. D. Brandling-Bennett, M. Beier, C. M. Mbogo, F. K. Atieli, M. L. Owaga, F. Juma and F. H. Collins (1993). "Confirmation that *Plasmodium falciparum* has aperiodic infectivity to *Anopheles gambiae*." Medical and Veterinary Entomology **7**(4): 373-376.
Goodspeed, D., E. W. Chehab, A. Min-Venditti, J. Braam and M. F. Covington (2012). "*Arabidopsis* synchronizes jasmonate-mediated defense with insect circadian behavior." Proceedings of the National Academy of Sciences **109**(12): 4674-4677.
Greischar, M. A., A. F. Read and O. N. Bjornstad (2014). "Synchrony in malaria infections: how intensifying within-host competition can be adaptive." American Naturalist **183**(2): E36-49.
Greischar, M. A., S. E. Reece, N. J. Savill and N. Mideo (2019). "The challenge of quantifying synchrony in malaria parasites." Trends in parasitology **35**(5): 341-355.
Häfker, N. S., B. Meyer, K. S. Last, D. W. Pond, L. Hüppe and M. Teschke (2017). "Circadian clock involvement in zooplankton diel vertical migration." Current Biology **27**(14): 2194-2201. e2193.
Hannon, E. R., D. M. Calhoun, S. Chadalawada and P. T. Johnson (2018). "Circadian rhythms of trematode parasites: applying mixed models to test underlying patterns." Parasitology **145**(6): 783-791.
Hardin, P. E. (2011). "Molecular genetic analysis of circadian timekeeping in *Drosophila*." Advances in genetics **74**: 141-173.
Hart, B. L. (1988). "Biological basis of the behavior of sick animals." Neuroscience & Biobehavioral Reviews **12**(2): 123-137.

Hawking, F. (1967). "The 24-hour periodicity of microfilariae: biological mechanisms responsible for its production and control." Proceedings of the Royal Society of London. Series B. Biological Sciences **169**(1014): 59-76.
Hawking, F. (1970). "The clock of the malaria parasite." Scientific American **222**(6): 123-131.
Hawking, F., M. J. Worms and K. Gammage (1968). "24-and 48-hour cycles of malaria parasites in the blood; their purpose, production and control." Transactions of the Royal Society of Tropical Medicine and Hygiene **62**(6): 731-738.
Hellweger, F. L., M. L. Jabbur, C. H. Johnson, E. van Sebille and H. Sasaki (2020). "Circadian clock helps cyanobacteria manage energy in coastal and high latitude ocean." The ISME journal **14**(2): 560-568.
Hevia, M. A., P. Canessa, H. Müller-Esparza and L. F. Larrondo (2015). "A circadian oscillator in the fungus *Botrytis cinerea* regulates virulence when infecting *Arabidopsis thaliana*." Proceedings of the National Academy of Sciences **112**(28): 8744-8749.
Hirako, I. C., P. A. Assis, N. S. Hojo-Souza, G. Reed, H. Nakaya, D. T. Golenbock, R. S. Coimbra and R. T. Gazzinelli (2018). "Daily Rhythms of TNFalpha Expression and Food Intake Regulate Synchrony of Plasmodium Stages with the Host Circadian Cycle." Cell Host Microbe **23**(6): 796-808 e796.
Horn, M., O. Mitesser, T. Hovestadt, T. Yoshii, D. Rieger and C. Helfrich-Förster (2019). "The circadian clock improves fitness in the fruit fly, *Drosophila melanogaster*." Frontiers in physiology **10**: 1374.
Hubbard, C. J., M. T. Brock, L. T. van Diepen, L. Maignien, B. E. Ewers and C. Weinig (2018). "The plant circadian clock influences rhizosphere community structure and function." The ISME journal **12**(2): 400-410.
Ingle, R. A., C. Stoker, W. Stone, N. Adams, R. Smith, M. Grant, I. Carre, L. C. Roden and K. J. Denby (2015). "Jasmonate signalling drives time-of-day differences in susceptibility of *Arabidopsis* to the fungal pathogen *Botrytis cinerea*." The Plant Journal **84**(5): 937-948.
Janse, C. J. and A. P. Waters (1995). "*Plasmodium berghei* - The Application Of Cultivation And Purification Techniques To Molecular Studies Of Malaria Parasites." Parasitology Today **11**(4): 138-143.
Johansson, M. and T. Köster (2019). "On the move through time–a historical review of plant clock research." Plant Biology **21**: 13-20.
Karunaweera, N. D., R. Carter, G. E. Grau, D. Kwiatkowski, G. Delgiudice and K. N. Mendis (1992). "Tumor Necrosis Factor-Dependent Parasite-Killing Effects During Paroxysms In Nonimmune *Plasmodium vivax* Malaria Patients." Clinical And Experimental Immunology **88**(3): 499-505.
Keller, M., J. Mazuch, U. Abraham, G. D. Eom, E. D. Herzog, H. D. Volk, A. Kramer and B. Maier (2009). "A circadian clock in macrophages controls inflammatory immune responses." Proceedings of the National Academy of Sciences of the United States of America **106**(50): 21407-21412.
Kennedy, F., E. Naylor and E. Jaramillo (2000). "Ontogenetic differences in the circadian locomotor activity rhythm of the talitrid amphipod crustacean *Orchestoidea tuberculata*." Marine biology **137**(3): 511-517.
Kiessling, S., G. Dubeau-Laramée, H. Ohm, N. Labrecque, M. Olivier and N. Cermakian (2017). "The circadian clock in immune cells controls the magnitude of *Leishmania* parasite infection." Scientific reports **7**(1): 10892.
Kim, Y. H. and M. A. Lazar (2020). "Transcriptional control of circadian rhythms and metabolism: A matter of time and space." Endocrine reviews **41**(5): 707-732.
Koilraj, A. J., V. K. Sharma, G. Marimuthu and M. Chandrashekaran (2000). "Presence of circadian rhythms in the locomotor activity of a cave-dwelling millipede *Glyphiulus cavernicolus sulu* (Cambalidae, Spirostreptida)." Chronobiology international **17**(6): 757-765.
Kondo, T. and M. Ishiura (2000). "The circadian clock of cyanobacteria." Bioessays **22**(1): 10-15.
Korgaonkar, N. S., A. Kumar, R. S. Yadav, D. Kabadi and A. P. Dash (2012). "Mosquito biting activity on humans & detection of *Plasmodium falciparum* infection in *Anopheles stephensi* in Goa, India." The Indian journal of medical research **135**(1): 120.

Koritala, B. S., C. Wager, J. C. Waters, R. Pachucki, B. Piccoli, Y. Feng, L. B. Scheinfeldt, S. M. Shende, S. Park and J. I. Hozier (2020). "Habitat-Specific Clock Variation and Its Consequence on Reproductive Fitness." Journal of biological rhythms **35**(2): 134-144.
Kumar, S., G. K. Christophides, R. Cantera, B. Charles, Y. S. Han, S. Meister, G. Dimopoulos, F. C. Kafatos and C. Barillas-Mury (2003). "The role of reactive oxygen species on *Plasmodium* melanotic encapsulation in *Anopheles gambiae*." Proceedings of the National Academy of Sciences **100**(24): 14139-14144.
Kwiatkowski, D. (1989). "Febrile temperatures can synchronize the growth of *Plasmodium falciparum in vitro*." J Exp Med **169**(1): 357-361.
Larrondo, L. F. and P. Canessa (2018). "The clock keeps on ticking: emerging roles for circadian regulation in the control of fungal physiology and pathogenesis." Fungal Physiology and Immunopathogenesis: 121-156.
Lee, J.-E. and I. Edery (2008). "Circadian regulation in the ability of *Drosophila* to combat pathogenic infections." Current biology **18**(3): 195-199.
Lu, D.-B., T.-P. Wang, J. W. Rudge, C. A. Donnelly, G.-R. Fang and J. P. Webster (2009). "Evolution in a multi-host parasite: chronobiological circadian rhythm and population genetics of *Schistosoma japonicum* cercariae indicates contrasting definitive host reservoirs by habitat." International journal for parasitology **39**(14): 1581-1588.
Lundy, S. R., T. Ahmad, T. Simoneaux, I. Benyeogor, Y. Robinson, Z. George, D. Ellerson, W. Kirlin, T. Omosun and F. O. Eko (2019). "Effect of time of day of infection on *Chlamydia* infectivity and pathogenesis." Scientific reports **9**(1): 1-12.
Magesa, S. M., Y. K. Mdira, J. A. Akida, I. C. Bygbjerg and P. H. Jakobsen (2000). "Observations on the periodicity of *Plasmodium falciparum* gametocytes in natural human infections." Acta Tropica **76**(3): 239-246.
Marcheva, B., K. M. Ramsey, E. D. Buhr, Y. Kobayashi, H. Su, C. H. Ko, G. Ivanova, C. Omura, S. Mo and M. H. Vitaterna (2010). "Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes." Nature **466**(7306): 627-631.
Martinaud, G., M. Billaudelle and J. Moreau (2009). "Circadian variation in shedding of the oocysts of *Isospora turdi* (Apicomplexa) in blackbirds (*Turdusmerula*): an adaptative trait against desiccation and ultraviolet radiation." International Journal for Parasitology **39**(6): 735-739.
Martinez-Bakker, M. and B. Helm (2015). "The influence of biological rhythms on host–parasite interactions." Trends in ecology & evolution **30**(6): 314-326.
Maywood, E., J. Chesham, N. Smyllie and M. Hastings (2014). "The Tau mutation of casein kinase 1ϵ sets the period of the mammalian pacemaker via regulation of Period1 or Period2 clock proteins." Journal of biological rhythms **29**(2): 110-118.
McKee, L., L. E. Johnson and R. Lange (1974). "Circadian variation in reticulocyte counts and immuno-detectable erythropoietin titers." Proceedings of the Society for Experimental Biology and Medicine **145**(4): 1284-1287.
McLean, K. J. and M. Jacobs-Lorena (2020). "The response of *Plasmodium falciparum* to isoleucine withdrawal is dependent on the stage of progression through the intraerythrocytic cell cycle." Malaria journal **19**(1): 1-9.
Mideo, N., S. E. Reece, A. L. Smith and C. J. Metcalf (2013). "The Cinderella syndrome: why do malaria-infected cells burst at midnight?" Trends in Parasitology **29**(1): 10-16.
Mons, B., C. J. Janse, E. G. Boorsma and H. J. Vanderkaay (1985). "Synchronized Erythrocytic Schizogony and Gametocytogenesis of *Plasmodium berghei in vivo and in vitro*." Parasitology **91**(DEC): 423-430.
Murdock, C. C., L. L. Moller-Jacobs and M. B. Thomas (2013). "Complex environmental drivers of immunity and resistance in malaria mosquitoes." Proceedings of the Royal Society B: Biological Sciences **280**(1770): 20132030.

Nguyen, K. D., S. J. Fentress, Y. Qiu, K. Yun, J. S. Cox and A. Chawla (2013). "Circadian gene Bmal1 regulates diurnal oscillations of Ly6Chi inflammatory monocytes." Science **341**(6153): 1483-1488.
O'Donnell, A. J., N. Mideo and S. E. Reece (2014). "Correction: disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated." Malaria Journal **13**(1): 503.
O'Donnell, A. J., K. F. Prior and S. E. Reece (2020). "Host circadian clocks do not set the schedule for the within-host replication of malaria parasites." Proceedings of the Royal Society B: Biological Sciences **287**(1932): 20200347.
O'Neill, J. S. and A. B. Reddy (2011). "Circadian clocks in human red blood cells." Nature **469**(7331): 498-503.
O'Donnell, A. J., M. A. Greischar and S. E. Reece (2021). "Mistimed malaria parasites re-synchronize with host feeding-fasting rhythms by shortening the duration of intra-erythrocytic development." Parasite Immunology **n/a**(n/a): e12898.
Olszewski, K. L. and M. Llinás (2011). "Central carbon metabolism of *Plasmodium* parasites." Molecular and biochemical parasitology **175**(2): 95-103.
Ouyang, Y., C. R. Andersson, T. Kondo, S. S. Golden and C. H. Johnson (1998). "Resonating circadian clocks enhance fitness in cyanobacteria." Proceedings of the National Academy of Sciences of the United States of America **95**(15): 8660-8664.
Owolabi, A. T., S. E. Reece and P. Schneider (2021). "Daily rhythms of both host and parasite affect antimalarial drug efficacy." Evolution, Medicine, and Public Health.
Pfenning-Butterworth, A., R. Cooper and C. Cressler (2021). "Daily feeding rhythm linked to microbiome composition in two zooplankton species."
Pigeault, R., Q. Caudron, A. Nicot, A. Rivero and S. Gandon (2018). "Timing malaria transmission with mosquito fluctuations." Evolution Letters.
Pittayakanchit, W., Z. Lu, J. Chew, M. J. Rust and A. Murugan (2018). "Biophysical clocks face a trade-off between internal and external noise resistance." Elife **7**: e37624.
Pittendrigh, C. S. and D. H. Minis (1972). "Circadian systems: longevity as a function of circadian resonance in *Drosophila melanogaster*." Proceedings of the National Academy of Sciences **69**(6): 1537-1539.
Platt, T. R., E. Graf, A. Kammrath and D. A. Zelmer (2010). "Diurnal migration of *Echinostoma caproni* (Digenea: Echinostomatidae) in ICR mice." Journal of Parasitology **96**(6): 1072-1075.
Prior, K., B. Middleton, A. Owolabi, M. Westwood, J. Holland, A. O'Donnell, M. Blackman, D. Skene and S. Reece (2021). "Synchrony between daily rhythms of malaria parasites and hosts is driven by an essential amino acid" Wellcome Open Research **6**(186).
Prior, K. F. (2018). The evolutionary ecology of circadian rhythms in malaria parasites, University of Edinburgh.
Prior, K. F., A. J. O'Donnell, S. S. Rund, N. J. Savill, D. R. Van Der Veen and S. E. Reece (2019). "Host circadian rhythms are disrupted during malaria infection in parasite genotype-specific manners." Scientific reports **9**(1): 1-12.
Prior, K. F., F. Rijo-Ferreira, P. A. Assis, I. C. Hirako, D. R. Weaver, R. T. Gazzinelli and S. E. Reece (2020). "Periodic Parasites and Daily Host Rhythms." Cell Host & Microbe **27**(2): 176-187.
Prior, K. F., D. R. van der Veen, A. J. O'Donnell, K. Cumnock, D. Schneider, A. Pain, A. Subudhi, A. Ramaprasad, S. S. C. Rund, N. J. Savill and S. E. Reece (2018). "Timing of host feeding drives rhythms in parasite replication." Plos Pathogens **14**(2).
Rawlinson, K. A., A. J. Reid, Z. Lu, P. Driguez, A. Wawer, A. Coghlan, G. Sankaranarayanan, S. K. Buddenborg, C. L. Diaz-Soria and C. McCarthy (2021). "Daily rhythms in the transcriptomes of the human parasite *Schistosoma mansoni.*" bioRxiv.

Reece, S. E., E. Ali, P. Schneider and H. A. Babiker (2010). "Stress, drugs and the evolution of reproductive restraint in malaria parasites." Proceedings of the Royal Society B: Biological Sciences **277**(1697): 3123-3129.
Reece, S. E., D. R. Drew and A. Gardner (2008). "Sex ratio adjustment and kin discrimination in malaria parasites." Nature **453**(7195): 609-614.
Reece, S. E., K. F. Prior and N. Mideo (2017). "The Life and Times of Parasites: Rhythms in Strategies for Within-host Survival and Between-host Transmission." Journal of Biological Rhythms **32**(6): 516-533.
Rijo-Ferreira, F., V. A. Acosta-Rodriguez, J. H. Abel, I. Kornblum, I. Bento, G. Kilaru, E. B. Klerman, M. M. Mota and J. S. Takahashi (2020). "The malaria parasite has an intrinsic clock." Science **368**(6492): 746-753.
Rijo-Ferreira, F., D. Pinto-Neves, N. L. Barbosa-Morais, J. S. Takahashi and L. M. Figueiredo (2017). "*Trypanosoma brucei* metabolism is under circadian control." Nature microbiology **2**(6): 1-10.
Rijo-Ferreira, F., J. S. Takahashi and L. M. Figueiredo (2017). "Circadian rhythms in parasites." PLoS Pathog **13**(10): e1006590.
Rojas, M. O. and M. Wasserman (1993). "Effect of low temperature on the in vitro growth of *Plasmodium falciparum*." Journal of Eukaryotic Microbiology **40**(2): 149-152.
Rubin, M. J., M. T. Brock, A. M. Davis, Z. M. German, M. Knapp, S. M. Welch, S. L. Harmer, J. N. Maloof, S. J. Davis and C. Weinig (2017). "Circadian rhythms vary over the growing season and correlate with fitness components." Molecular ecology **26**(20): 5528-5540.
Rund, S. S., J. E. Gentile and G. E. Duffield (2013). "Extensive circadian and light regulation of the transcriptome in the malaria mosquito *Anopheles gambiae*." BMC Genomics **14**: 218.
Rund, S. S. C., A. J. O'Donnell, J. E. Gentile and S. E. Reece (2016). "Daily Rhythms in Mosquitoes and Their Consequences for Malaria Transmission." Insects **7**(2).
Schaer, J., S. L. Perkins, J. Decher, F. H. Leendertz, J. Fahr, N. Weber and K. Matuschewski (2013). "High diversity of West African bat malaria parasites and a tight link with rodent *Plasmodium* taxa." Proceedings of the National Academy of Sciences **110**(43): 17415-17419.
Scheiermann, C., J. Gibbs, L. Ince and A. Loudon (2018). "Clocking in to immunity." Nature Reviews Immunology **18**(7): 423-437.
Scheiermann, C., Y. Kunisaki and P. S. Frenette (2013). "Circadian control of the immune system." Nature Reviews Immunology **13**(3): 190-198.
Schneider, P., S. S. C. Rund, N. L. Smith, K. F. Prior, A. J. O'Donnell and S. E. Reece (2018). "Adaptive periodicity in the infectivity of malaria gametocytes to mosquitoes." Proceedings of the Royal Society B-Biological Sciences **285**(1888).
Schuster, F. L. (2002). "Cultivation of *Plasmodium* spp." Clinical Microbiology Reviews **15**(3): 355-364.
Sharma, V. K. (2003). "Adaptive significance of circadian clocks." Chronobiology International **20**(6): 901-919.
Sheeba, V., V. Sharma, M. Chandrashekaran and A. Joshi (1999). "Persistence of eclosion rhythm in *Drosophila melanogaster* after 600 generations in an aperiodic environment." Naturwissenschaften **86**(9): 448-449.
Sinka, M., S. Pironon, N. Massey, J. Longbottom, J. Hemingway, C. Moyes and K. Willis (2020). "A new malaria vector in Africa: predicting the expansion range of *Anopheles stephensi* and identifying the urban populations at risk." Proceedings of the National Academy of Sciences **117**(40): 24900-24908.
Smith, L. M., F. C. Motta, G. Chopra, J. K. Moch, R. R. Nerem, B. Cummins, K. E. Roche, C. M. Kelliher, A. R. Leman and J. Harer (2020). "An intrinsic oscillator drives the blood stage cycle of the malaria parasite *Plasmodium falciparum.*" Science **368**(6492): 754-759.
Sojka, P., R. Griess and M. Nielsen (2013). "Locomotor activity and body temperature in selected mouse lines differing greatly in feed intake." Journal of animal science **91**(8): 3557-3563.

Sougoufara, S., S. M. Diédhiou, S. Doucouré, N. Diagne, P. M. Sembène, M. Harry, J.-F. Trape, C. Sokhna and M. O. Ndiath (2014). "Biting by *Anopheles funestus* in broad daylight after use of long-lasting insecticidal nets: a new challenge to malaria elimination." Malaria journal **13**(1): 1-7.
Spence, P. J., W. Jarra, P. Lévy, A. J. Reid, L. Chappell, T. Brugat, M. Sanders, M. Berriman and J. Langhorne (2013). "Vector transmission regulates immune control of *Plasmodium* virulence." Nature **498**(7453): 228-231.
Spoelstra, K., M. Wikelski, S. Daan, A. S. Loudon and M. Hau (2016). "Natural selection against a circadian clock gene mutation in mice." Proceedings of the National Academy of Sciences **113**(3): 686-691.
Stephens, R., R. L. Culleton and T. J. Lamb (2012). "The contribution of *Plasmodium chabaudi* to our understanding of malaria." Trends in parasitology **28**(2): 73-82.
Stone, E. F., B. O. Fulton, J. S. Ayres, L. N. Pham, J. Ziauddin and M. M. Shirasu-Hiza (2012). "The circadian clock protein timeless regulates phagocytosis of bacteria in *Drosophila*." PLoS Pathog **8**(1): e1002445.
Stroebel, A. M., M. Bergner, U. Reulbach, T. Biermann, T. W. Groemer, I. Klein and J. Kornhuber (2010). "Statistical methods for detecting and comparing periodic data and their application to the nycthemeral rhythm of bodily harm: A population based study." Journal of circadian rhythms **8**(1): 1-10.
Su, J., F. Zhou and D.-B. Lu (2013). "A circular analysis of chronobiology of *Schistosoma japonicum* cercarial emergence from hilly areas of Anhui, China." Experimental parasitology **135**(2): 421-425.
Subudhi, A. K., A. J. O'Donnell, A. Ramaprasad, H. M. Abkallo, A. Kaushik, H. R. Ansari, A. M. Abdel-Haleem, F. Ben Rached, O. Kaneko, R. Culleton, S. E. Reece and A. Pain (2020). "Malaria parasites regulate intra-erythrocytic development duration via serpentine receptor 10 to coordinate with host rhythms." Nature Communications **11**(1): 2763.
Takahashi, J. S. (2017). "Transcriptional architecture of the mammalian circadian clock." Nature Reviews Genetics **18**(3): 164-179.
Taliaferro, L. G. (1928). "Return to normal of the asexual cycle in bird malaria after retardation by low temperatures in vitro." The Journal of Preventive Medicine **2**: 525-541.
Teuscher, F., M. L. Gatton, N. Chen, J. Peters, D. E. Kyle and Q. Cheng (2010). "Artemisinin-induced dormancy in *Plasmodium falciparum*: duration, recovery rates, and implications in treatment failure." J Infect Dis **202**(9): 1362-1368.
Thomsen, E. K., G. Koimbu, J. Pulford, S. Jamea-Maiasa, Y. Ura, J. B. Keven, P. M. Siba, I. Mueller, M. W. Hetzel and L. J. Reimer (2017). "Mosquito behavior change after distribution of bednets results in decreased protection against malaria exposure." The Journal of infectious diseases **215**(5): 790-797.
Tinbergen, J. and S. Daan (1979). "Young guillemots (*Uria lomvia*) leaving their Arctic breeding cliffs: A daily rhythm in numbers and risk."
Trail, D. R. S. (1980). "Behavioral interactions between parasites and hosts: host suicide and the evolution of complex life cycles." The American Naturalist **116**(1): 77-91.
Wamae, P. M., A. K. Githeko, G. O. Otieno, E. W. Kabiru and S. O. Duombia (2015). "Early biting of the *Anopheles gambiae s.s.* and its challenges to vector control using insecticide treated nets in western Kenya highlands." Acta Tropica **150**: 136-142.
Westwood, M. L., A. J. O'Donnell, C. de Bekker, C. M. Lively, M. Zuk and S. E. Reece (2019). "The evolutionary ecology of circadian rhythms in infection." Nature ecology & evolution: 1.
Westwood, M. L., A. J. O'Donnell, P. Schneider, G. F. Albery, K. F. Prior and S. E. Reece (2020). "Testing possible causes of gametocyte reduction in temporally out-of-synch malaria infections." Malaria journal **19**(1): 1-10.
Yohannes, M. and E. Boelee (2012). "Early biting rhythm in the afro-tropical vector of malaria, *Anopheles arabiensis*, and challenges for its control in Ethiopia." Medical and veterinary entomology **26**(1): 103-105.

Yon, F., D. Kessler, Y. Joo, S. G. Kim and I. T. Baldwin (2017). "Fitness consequences of a clock pollinator filter in *Nicotiana attenuata* flowers in nature." Journal of integrative plant biology **59**(11): 805-809.
Zhang, R., N. F. Lahens, H. I. Ballance, M. E. Hughes and J. B. Hogenesch (2014). "A circadian gene expression atlas in mammals: implications for biology and medicine." Proceedings of the National Academy of Sciences **111**(45): 16219-16224.
Zhang, Z., H.-J. Wang, D.-R. Wang, W.-M. Qu and Z.-L. Huang (2017). "Red light at intensities above 10 lx alters sleep–wake behavior in mice." Light: Science & Applications **6**(5): e16231-e16231.
Zhuang, X., D. Forde, S. Tsukuda, V. D'Arienzo, L. Mailly, J. M. Harris, P. A. Wing, H. Borrmann, M. Schilling and A. Magri (2021). "Circadian control of hepatitis B virus replication." Nature communications **12**(1): 1-13.
Zhuang, X., A. Magri, M. Hill, A. G. Lai, A. Kumar, S. B. Rambhatla, C. L. Donald, A. F. Lopez-Clavijo, S. Rudge and K. Pinnick (2019). "The circadian clock components BMAL1 and REV-ERBα regulate flavivirus replication." Nature communications **10**(1): 1-13.
Zhuang, X., S. Tsukuda, F. Wrensch, P. A. Wing, H. Borrmann, J. M. Harris, S. B. Morgan, L. Mailly, N. Thakur and C. Conceicao (2021). "Circadian regulation of SARS-CoV-2 infection in lung epithelial cells." bioRxiv.

Appendix 1

I have contributed to these publications in the following manner:

Contributed considerably to designing and carrying out experimental work, interpretation of results and writing of the manuscript:

1. Subudhi A. K., **O'Donnell A. J.**, Ramaprasad A., Abkallo H. M., Kaushik A., Ansari H. R., Abdel-Haleem A. M., Rached F. B., Kaneko O., Culleton R., Reece S. E. & Pain, A. (2020). Malaria parasites regulate intra-erythrocytic development duration via serpentine receptor 10 to coordinate with host rhythms. Nature Communications 11(2763).

   Investigates rhythmicity in parasite genes in infections both matched and mismatched to host rhythms revealing a role of SR10 in determining IDC length and providing evidence for parasite control of their IDC rhythms.

2. Westwood M. L., **O'Donnell A. J.**, Schneider P., Albery G. F., Prior K. F., & Reece S. E. (2020). Testing possible causes of gametocyte reduction in temporally out-of-synch malaria infections. Malaria Journal, 19(1):1-10.

   Tests whether the observed 50% reduction in gametocytes in temporally mismatched parasites can be explained by a reduction in conversion rate or increased clearance by the hosts immune responses

3. Rund S. S. C., **O'Donnell A. J.**, Gentile J. E. & Reece, S. E. (2016) Daily Rhythms in Mosquitoes and Their Consequences for Malaria Transmission. Insects, 7(2):14.

   Outlines new hypotheses for how daily rhythms in mosquitoes affects their capacity to transmit malaria parasites.

Contributed experimental support, and interpretation and editing of the manuscript:

4. Prior K. F., Middleton B., Owolabi A. T. Y., Westwood M. L., Holland, J., **O'Donnell A. J.**, Blackman M. J., Skene D. J., Reece S. E. (2021) Synchrony between daily rhythms of malaria parasites and hosts is driven by an essential amino acid. Wellcome Open Research 2021, 6:186

   A large scale metabolomics screen for rhythmic compounds in infected hosts. Identifies a candidate timing cue, Isoleucine, that parasites may use to coordinate their development schedule with the host and tests the influence of this amino acid on parasite development in vitro

5. Prior K. F., **O'Donnell A. J.**, Rund S. S., Savill N. J., van der Veen D. R., & Reece S. E. (2019). Host circadian rhythms are disrupted during malaria infection in parasite genotype-specific manners. Scientific Reports, 9:10905.

   Characterises differences in infection dynamics of three parasite genotypes in response to timing disruption and shows that the disruption is a genetically variable virulence trait

6. Schneider P., Rund S. S. C., Smith N. L., Prior K. F., **O'Donnell A. J.**, & Reece S. E. (2018). Adaptive periodicity in the infectivity of malaria gametocytes to mosquitoes. Proceedings of the Royal Society B, 285(1888):20181876.

   Demonstrates that at night, gametocytes are twice as infective to mosquitoes, despite being less numerous in the blood. Enhanced parasite infectiousness at night interacts with mosquito circadian rhythms to increase sporozoite burdens four-fold when mosquitoes become infected from feeding during their rest phase.

7. Prior K. F., van der Veen D. R., **O'Donnell A. J.**, Cumnock K., Schneider D., Pain A., Subudhi A., Ramaprasad A., Rund S. S. C., Savill N. J., & Reece S. E. (2018) Timing of host feeding drives rhythms in parasite replication. PLOS Pathogens, 14(2):e1006900.

   Reveals that the hosts' peripheral rhythms (associated with the timing of feeding and metabolism), but not rhythms driven by the central, light-entrained circadian oscillator in the brain, determine the timing (phase) of parasite rhythms.

Contributed experimental samples and/or editing support for the manuscript:

8. Davidson M. S., Yahiya S., Chmielewski J., **O'Donnell A. J.**, Gurung P., Jeninga M., Prommana P., Andrew D., Petter M, Uthaipibull C., Boyle M., Ashdown G. W., Dvorin J. D., Reece S. E., Wilson D. W., Ando D. M., Dimon M. & Baum J. (2021). Automated detection and staging of malaria parasites from cytological smears using convolutional neural networks. medRxiv.

   Describes a novel machine learning method for identifying and staging parasites from microscopy images.

9. Birget P. L. G., Schneider P., **O'Donnell A. J.**, & Reece S. E. (2019). Adaptive phenotypic plasticity in malaria parasites is not constrained by previous responses to environmental change. Evolution, Medicine, and Public Health, eoz028.

   Malaria parasites have evolved flexible strategies to cope with the changing conditions they experience during infections. We show that using such flexible strategies does not impact upon the parasites' ability to grow (resulting in disease symptoms) or transmit (spreading the disease).

10. Westwood M. L., **O'Donnell A. J.**, de Bekker C., Lively C. M., Zuk M., & Reece, S. E. (2019). The evolutionary ecology of circadian rhythms in infection. Nature ecology & evolution, 18(1).

    Explores how hosts use rhythms to defend against infection, why parasites have rhythms and whether parasites can manipulate host clocks to their own ends.

11. Lippens C., Guivier E., Reece S. E., **O'Donnell A. J.**, Cornet S., Faivre B., & Sorci G. (2018). Early Plasmodium-induced inflammation does not accelerate aging in mice. Evolutionary Applications, 12(2):314-23.

Tests the hypothesis that early activation of the inflammatory response confers protection against infection, it results in reduced reproductive output at old age and shortened longevity.

12. Birget P. L., Repton C., **O'Donnell A. J.**, Schneider P., & Reece S. E. (2017) Phenotypic plasticity in reproductive effort: malaria parasites respond to resource availability. Proceedings of the Royal Society B, 284(1860):20171229.

    Reveals that the malaria parasite Plasmodium chabaudi responds to host anaemia by increasing investment in transmission stages because, counterintuitively, host anaemia represents a better environment for parasite replication. Furthermore, evolutionary potential in form of genetic variation exists in the extent that parasite strains respond to changes in red blood cell resources.

13. Schneider P., Bell A. S., Sim D. G., **O'Donnell A. J.**, Blanford S., Paaijmans K. P., Read A. F. and Reece S. E. (2012) Virulence, drug sensitivity and transmission success in the rodent malaria, *Plasmodium chabaudi*. Proceedings of the Royal Society B. 279(1747):4677-85.

    Drug treatment selects for the evolution of more harmful parasite strains.

Paper #1 (2021): Fitness costs of disrupting circadian rhythms in malaria parasites

PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2011) **278**, 2429–2436
doi:10.1098/rspb.2010.2457
Published online 5 January 2011

Fitness costs of disrupting circadian rhythms in malaria parasites

Aidan J. O'Donnell[1,*], Petra Schneider[2], Harriet G. McWatters[3,†] and Sarah E. Reece[1,2,†]

[1]*Institute of Evolution, Institute of Immunology and Infection Research, and* [2]*Centre for Immunity, Infection and Evolution, University of Edinburgh, Edinburgh EH9 3JT, UK*
[3]*Department of Plant Sciences, Oxford University, Oxford OX1 3RB, UK*

Circadian biology assumes that biological rhythms maximize fitness by enabling organisms to coordinate with their environment. Despite circadian clocks being such a widespread phenomenon, demonstrating the fitness benefits of temporal coordination is challenging and such studies are rare. Here, we tested the consequences—for parasites—of being temporally mismatched to host circadian rhythms using the rodent malaria parasite, *Plasmodium chabaudi*. The cyclical nature of malaria infections is well known, as the cell cycles across parasite species last a multiple of approximately 24 h, but the evolutionary explanations for periodicity are poorly understood. We demonstrate that perturbation of parasite rhythms results in a twofold cost to the production of replicating and transmission stages. Thus, synchronization with host rhythms influences in-host survival and between-host transmission potential, revealing a role for circadian rhythms in the evolution of host–parasite interactions. More generally, our results provide a demonstration of the adaptive value of circadian rhythms and the utility of using an evolutionary framework to understand parasite traits.

Keywords: cell cycle; plasticity; periodicity; synchronicity; biological rhythms; *Plasmodium chabaudi*

1. INTRODUCTION

Circadian clocks underlie biological rhythms with a periodicity of approximately 24 h across a range of taxa, spanning from bacteria to plants, insects and vertebrates. All levels of biological organization within an organism, from gene expression to immune function, behaviour and seasonal patterns of reproduction are subject to regulation by the clock [1]. A cornerstone of chronobiology is the idea that organisms have evolved circadian clocks to allow coordination of physiology and behaviour with the Earth's daily rotation [2]. Despite circadian clocks being such a widespread phenomenon, demonstrating the fitness benefits of this coordination is challenging. Currently, the clearest evidence comes from experiments showing that having a circadian clock, the periodicity of which resonates with that of the environment, enhances the competitive ability of cyanobacteria [3] and plants [4], and larval growth rate in insects [5]. While considerable circumstantial evidence suggests that a circadian clock enhances fitness [6], studies unequivocally testing the adaptive significance of clocks are scarce for two reasons. First, the majority of recent research in the field of chronobiology has focused on asking questions about clock mechanisms [7–11]. Second, it is very difficult to do laboratory experiments that perturb timing schedules in ecologically realistic ways, and controlling for potentially confounding effects in field studies is equally challenging [12].

Here, we test whether matching developmental schedules to time of day affects the growth and transmission potential of malaria (*Plasmodium*) parasites. Malaria parasites replicate asexually in a vertebrate host and sexually in the mosquito vector. During the night, at the end of the cell cycle, each mature parasite (termed schizont) synchronously releases multiple daughter progeny (termed merozoites). *Plasmodium* species that infect humans have synchronous cell-cycle durations of 48 or 72 h and cause recurrent fever every 2 or 3 days, which is sufficiently precise to be a diagnostic feature of the disease [13]. Both the evolutionary and mechanistic explanations of this periodicity are poorly understood, but that it is always a multiple of 24 h suggests that circadian clocks regulate parasite rhythms. Every cell cycle, a proportion of parasites differentiate into male and female stages (gametocytes), which reproduce sexually when taken up by a mosquito. Rapid asexual replication is central to establishing and maintaining infections; the production of gametocytes is essential for transmission between hosts [14]. Malaria parasites offer a useful system for circadian studies because asexual and sexual stages can be distinguished and precisely quantified using molecular techniques developed specifically for this purpose [15–17]. Also, parasites are engaged in a life or death struggle with their hosts—so if perturbation of their cell cycle alters important interactions with their in-host environment, it will result in immediate and ecologically relevant fitness consequences.

There is increasing interest in the reciprocal approach of using unicellular taxa to test the generality of evolutionary theories developed for multicellular taxa and using an evolutionary approach to understand the biology of important unicellular taxa [14,18–20]. Matching the host circadian rhythm appears to be achieved using output from host clocks as a time cue for scheduling

* Author for correspondence (aidan.odonnell@ed.ac.uk).
† These authors contributed equally to the work.

Received 10 November 2010
Accepted 8 December 2010

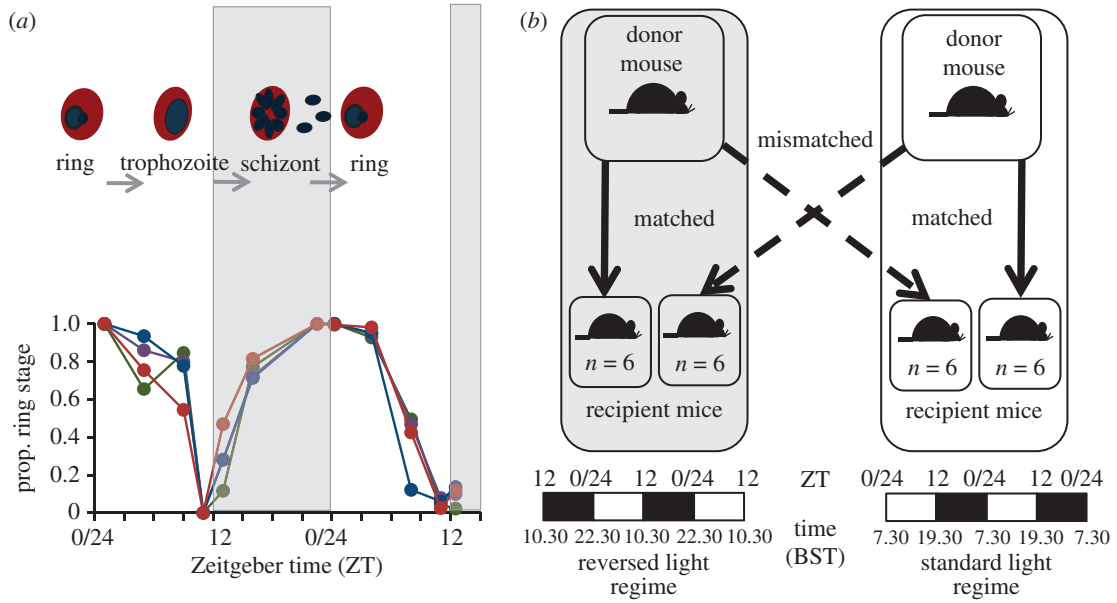

Figure 1. (*a*) The asexual cycle of *P. chabaudi* follows the pattern of day and night with ring stages being produced in the morning, which develop to trophozoites in the afternoon and release merozoites (progeny) from schizonts at night. Data are from four infections initiated and followed prior to our main experiment to verify that the *P. chabaudi* genotype used (AJ) has a synchronous and 24 h cell cycle. (*b*) The experiment was designed to test whether this temporal alignment is beneficial to parasite replication and transmission. Arrows indicate transfers of parasites to recipient hosts (four groups of six mice) within and between two rooms with different lighting schedules. Parasites remaining in the same room acted as controls as they were matched to host rhythms. Parasites transferred to hosts with a different rhythm from their donor were temporally mismatched, analogous to jetlag. Dark and light bars indicated lights-on/lights-off status throughout each 48 h period. Zeitgeber time (ZT) is displayed above the bars; ZT 0/24, time of lights-on and ZT 12, time of lights-off.

progression throughout the cell (replication) cycle. Previous work has demonstrated that if the rhythm of rodent malaria parasites is perturbed, it returns to match the host circadian rhythm within a few cell cycles [21–23]. Furthermore, human malaria parasites lose synchronicity in their cell cycle during *in vitro* culture [24], but the addition of melatonin appears to restore coordination [25]. Here, we show that perturbing the rhythm of parasites relative to the host body clock has consequences for their proliferation and transmission potential. Our study thus achieves a rare link between chronobiology and evolutionary biology, as well as representing a novel application of evolutionary theory to an organism of high medical importance.

2. MATERIAL AND METHODS

(a) *Parasites and hosts*

Hosts were 10–12-week-old MF1 male mice housed at 21°C with ad libitum food and drinking water supplemented with 0.05 per cent para-aminobenzoic acid (to supplement parasite growth). *Plasmodium chabaudi* has previously been reported to have a synchronous cell cycle of 24 h [26], but—prior to our main experiment—we set up infections to verify that this was also the case for the clone (AJ) used here. For this study, we initiated four replicate infections with 1×10^6 parasitized red blood cells (RBCs) in mice maintained on a 12 L : 12 D cycle. We followed the proportion of parasites at ring stage at approximately four-hourly intervals over 36 h on days 3 and 4 post-infection (pi). These data are presented in figure 1*a* and demonstrate unambiguously that the cell-cycle clone AJ is synchronous with a duration of 24 h.

Our main experiment required manipulating the circadian rhythms of hosts. We achieved this by housing mice in two rooms, each maintained on a 12 L : 12 D cycle, that differed only in the timing of 'lights-on'. In the 'standard schedule' room, lights were on during the day (lights-on: 07.30 h; lights-off: 19.30 h); in the 'light-reversed' room, lights were on during the night (lights-on 22.30 h; lights-off: 10.30 h). All mice in the experiment were allowed to acclimatize to their respective lighting regimes for two weeks before infection. This allowed mice time to entrain to their schedule, as previous work has demonstrated this occurs within 7 days [27]. However, prior to infecting the mice, we verified that they behaved as expected for their light : dark schedule, i.e. were active during the dark period and inactive when lights were on. In each room, a host was infected with 1×10^6 *P. chabaudi* (clone AJ) parasitized RBCs to provide 'donor' parasites to initiate experimental infections (figure 1*b*). Mice used in the experiment were housed in groups of three and a total of 24 were used ($n = 6$ infections per treatment group).

(b) *Experimental design*

Parasites at the ring stage from the donor infection in each room were used to infect hosts (with 1×10^6 parasitized RBCs) in both rooms (figure 1*b*). Parasites originating from the 'standard regime' room were collected at Zeitgeber time (ZT) 0 (the time of lights-on) and used to infect *simultaneously* mice in the same room and the light-reversed room. The same procedure was repeated 15 h later, at ZT 0 in the light-reversed room for parasites originating from this room, which were again used to infect *simultaneously* mice in the light-reversed room and the standard regime room. This produced two treatments: parasites 'matched' to the host rhythm (control infections; mice infected with parasites from the same room) and parasites 'mismatched' to the host rhythm (experimental infections; mice infected with parasites from the room on the opposite lighting schedule). Parasites in

the mismatched treatments thus underwent a temporal phase shift, analogous to the cross-continental travel that induces jet lag. This experimental design provides four cross-factored groups of infections (two schedules of origin × two destination schedules) and enables the performance of mismatched parasites' growth and transmission potential to be compared with those matched to host rhythms in both the original and the destination rooms.

(c) *Data collection*

All mice were sampled twice daily, in the morning at 09.00 h and in the evening at 19.00 h (BST), during the growth phase of infections (that is, from days 0–7 pi [28]). Focusing on the growth phase minimized the influence of potentially confounding variables, such as anaemia and immune responses, which significantly influence parasitaemia after peak, and avoided the risk of host mortality, causing an unbalanced design and reducing power. At each sampling point, thin smears were made, samples were taken to quantify gametocyte (10 µl) and total parasite (5 µl) densities, and RBC densities measured using flow cytometry (Beckman Coulter; for 09.00 h samples only). Thin smears were made from tail blood and stained with 20 per cent Giemsa buffered in 80 per cent phosphate buffer solution for 20 min. These smears were used to determine the cell-cycle stage of parasites; 200 parasites per smear were examined and each classified as one of the following stages: ring, trophozoite, schizont or gametocyte [26]. In the few smears where the parasitaemia was very low, only 100 (or in very rare cases 50) parasites were examined. The densities of gametocytes and total parasites were measured using reverse transcriptase–quantitative PCR (RT–qPCR) and qPCR, respectively. Blood samples were taken for DNA (5 µl) and for RNA (10 µl); DNA and RNA were extracted using the ABI Prism 6100 and cDNA was obtained from RNA using the high-capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's protocol [17]. Total parasite and gametocyte densities were obtained using primers based on the gametocyte-expressed gene PC302249.00.0 [29]. This protocol was applied to DNA to give a density of total parasites (qPCR) and also on cDNA to count gametocyte density (RT–qPCR) specifically. We counted total parasites from samples taken on days 1, 3, 5 and 7 and we counted gametocytes from all samples (days 1–7).

(d) *Statistical analysis*

We used R v. 2.6.1 (The R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org) for all analyses. To verify the validity of the methods we used, several checks were necessary: specifically, that (1) sequestration and (2) qPCR assays did not bias density estimates when parasites were sampled late in the cell cycle. For these analyses, we investigated whether: (1) parasite densities differed between early and late sampling points within the same cell cycle owing to sequestration of trophozoite and/or schizont stages and (2) parasite densities, estimated by qPCR and smear from the same set of samples, differed according to the method used. As these analyses involved paired data, we used one-sample *t*-tests to test whether the mean differences between pairs of (1) early/late samples and (2) qPCR/smear estimates were significantly different from zero. Having verified our methods, we then used general linear models to analyse our experimental data by testing whether: (3) timing of the parasite cell cycle of matched (control) infections differed between rooms; (4) parasites in the matched groups differed in performance across the rooms; and (5) there were consequences of being matched or mismatched to host rhythm for total parasite and gametocyte densities. The details of each analysis are explained below.

(i) *Validation of experimental procedures*

(1) *Sequestration during development*. We tested the possibility that sequestration of parasites in late stages of development (trophozoites and schizonts [30,31]) could bias estimates of parasite density and developmental stage using the matched (control) treatments. If late-stage parasites sequester, parasitaemia estimates from blood smears will appear to be lower in samples taken at the dark period late in the cell cycle compared with those taken during the light period when parasites are at ring or early trophozoite stages. There was no significant difference in the parasitaemia of each infection between subsequent sample points on day 3 (60–72 h pi for the light-reversed room and 72–84 h pi for the standard regime room; $t = 2.05$; $p = 0.065$), suggesting that sequestration does not significantly bias parasite estimates from samples taken later in the cell cycle.

(2) *Assays for parasite density.* We investigated the possibility that qPCR (which counts genomes) could overestimate parasite density in samples taken late in the cell cycle when mitotic division during maturation into schizonts may have begun. To test whether qPCR overestimates parasite density (i.e. the number of infected RBCs per millilitre) relative to estimates from blood smears, we examined whether the difference in density estimates from qPCR and blood smears changes throughout the cell cycle (i.e. do the estimates from qPCR increase throughout the cell cycle more than estimates from smears?). Specifically, we compared the change in densities between samples taken on day 3 pi early and late in the same cell cycle (i.e. 72 h pi for the light-reversed room and 84 h pi for the standard schedule room) for all infections and found no significant difference ($t = 1.66$; $p = 0.11$). Furthermore, we examined blood smears from each infection from days 3 to 7 to investigate whether schizonts were present in the circulation and, if so, whether their prevalence increased as infections progressed. The average number of schizonts observed each day, in approximately 3500 red blood cells, ranged from 1.5 to 2.1 and did not show any temporal trends or variation across treatment groups. Therefore, the very low prevalence of schizonts in blood smears suggests that the potential inaccuracies of qPCR (by falsely counting multiple genomes within a single schizont) are negligible.

(ii) *Experiment*

(3) *Schedule manipulations.* To verify the experimental manipulations had been successful at creating different parasite schedules in each room, the developmental stages of parasites in matched (control) infections in the standard schedule room were compared with matched (control) infections in the light-reversed room. We compared these parasite schedules at 60 h pi as this was the earliest sampling point when sufficient parasites could be detected for staging by microscopy.

(4) *Performance of matched infections*. To test whether the matched (control) groups differed in performance across the light-reversed and standard schedule rooms, we compared the overall performance of these groups. Specifically,

we compared cumulative parasite densities produced by infections in each matched group. A significant difference between the two matched groups would suggest that a room-of-origin effect carried over into the experimental infections and invalidated comparisons between mismatched infections and matched infections in the same destination room.

(5) *Effects of mismatch to host circadian rhythm*. To investigate the effects of mismatch on the densities of total parasites and gametocytes, both the treatment (matched or mismatched) and the light regime (standard or light-reversed schedule) and their interaction were fitted and models simplified using stepwise deletion [32]. We used the cumulative densities of parasites or gametocytes, calculated for each infection from days 1 to 7 pi. As infections originating from the standard schedule room are ahead of infections originating from the light-reversed room, the same *duration of infection* occurs in the morning for infections from the standard schedule room and in the evening for infections from the light-reversed room. Therefore, for the analysis of total parasite densities, we have four sampling points for each treatment group, where the duration of infections is consistent because we counted parasites every other day from the initiation of the infections. These points occur at days 1, 3, 5 and 7 pi (durations of 24, 72, 120 and 168 h pi), being the samples collected at 09.00 h for parasites originating from the standard schedule room and at 19.00 h for parasites from the light-reversed room.

In contrast to counts of total parasites, gametocyte densities are several orders of magnitude lower. To maximize our power, we counted gametocytes from all sampling time points (days 1–7 pi; 12–168 h pi), and all samples contributed to the cumulative density for each infection. We also tested whether our match/mismatch treatment influenced how much variation in the synchronicity of cell-cycle schedules occurs within infections. We compared the coefficient of variation (standard deviation of the mean) for the proportion of ring-stage parasites observed in blood films throughout infections (days 3–7 pi; 12–168 h pi) in the matched and mismatched groups.

3. RESULTS

(a) *Entrainment of parasite rhythms*

Our 'jetlag' experimental design required that infections in the two rooms had different phases relative to each other. To verify that the phase of the cell-cycle rhythm was set by the lighting schedule, the developmental stage of parasites was examined when parasites in each treatment reached 60 hpi. At 60 hpi in the light-reversed room, lights were on and parasites were expected to be at the ring stage, whereas in the standard schedule room, lights were off and so parasites should be at later cell-cycle stages. As expected, a greater proportion of ring-stage parasites was observed in infections originating from the light-reversed room than the standard schedule room ($F_{1,17} = 12.29$; $p = 0.003$), demonstrating that parasites originating from each room had differently phased cell-cycle rhythms.

We also tested whether there was a significant difference in the performance of infections in the two matched (control) groups, which would suggest that a room-of-origin effect carried over into the experimental infections. However, the cumulative parasite densities of infections in these groups did not differ significantly ($F_{1,10} = 1.40$; $p = 0.265$), revealing that the matched infections performed similarly, regardless of their lighting schedule. This enables the performance of mismatched parasites to be compared with both groups of matched infections (i.e. to matched infections in the same destination room, as well as matched infections remaining in the room of origin).

(b) *Effects of mismatch to host circadian rhythm*

There was a strong effect of perturbing parasite cell cycle relative to the host rhythm (figure 2). We found significant effects of our mismatch/match treatment on the production of total parasites and gametocytes, but neither the original schedule nor its interaction with treatment significantly influenced infections (table 1). The cumulative parasite densities (figure 2*a*, upper panel) of matched infections were double those of mismatched infections ($F_{1,22} = 8.38$; $p = 0.008$; matched mean $= 1.7 \pm 0.25 \times 10^9$ ml^{-1}; mismatched $= 0.85 \pm 0.15 \times 10^9$ ml^{-1}). Cumulative gametocyte densities (figure 2*b*, upper panel) followed the same pattern, in which mismatched infections produced significantly fewer gametocytes ($F_{1,22} = 6.84$; $p = 0.016$; matched mean $= 1.9 \pm 0.21 \times 10^5$ gametocytes ml^{-1}; mismatched $= 1.01 \pm 0.21 \times 10^5$ ml^{-1}). The cumulative densities (upper panels) are decomposed into their temporal dynamics (lower panels) in figure 2. All *F*-ratios and *p*-values are given in table 1, along with the mean differences in the cumulative densities of mismatch and matched infections.

Previous studies suggest that, when perturbed, parasite rhythms change to re-align with the host rhythm. Therefore, we hypothesized that as parasites adjust to their new environment, the schedules of mismatched parasites should become increasingly different from those of matched infections remaining on the original schedule, and increasingly similar to the schedules of matched infections in the same destination room. This adjustment is predicted to cause greater variation between the schedules of mismatched and matched infections, and this is reflected by significantly greater coefficients of variation in mismatched than in matched parasites ($F_{1,22} = 4.69$; $p = 0.041$). Furthermore, neither the original schedule nor its interaction with treatment significantly influenced the extent of synchronicity in cell-cycle schedules (table 1).

4. DISCUSSION

Our data provide a rare demonstration of the impact that circadian rhythms have on fitness. Specifically, we reveal that parasites forced out of synchrony with the host's schedule paid substantial costs, as a single phase shift reduced both in-host replication and the production of transmission stages by around 50 per cent. These costs are likely to have broad implications for parasite survival and reproduction. Malaria parasites must optimize the trade-off between investment in replication for in-host survival and the production of gametocytes for between-host transmission. Parasites with low replication rates are vulnerable to clearance by the immune system, antimalarial drugs and are poor competitors in genetically mixed infections [33–37]. For example, subtle differences in the replication rate of co-infecting strains can lead to substantial competitive suppression in mixed

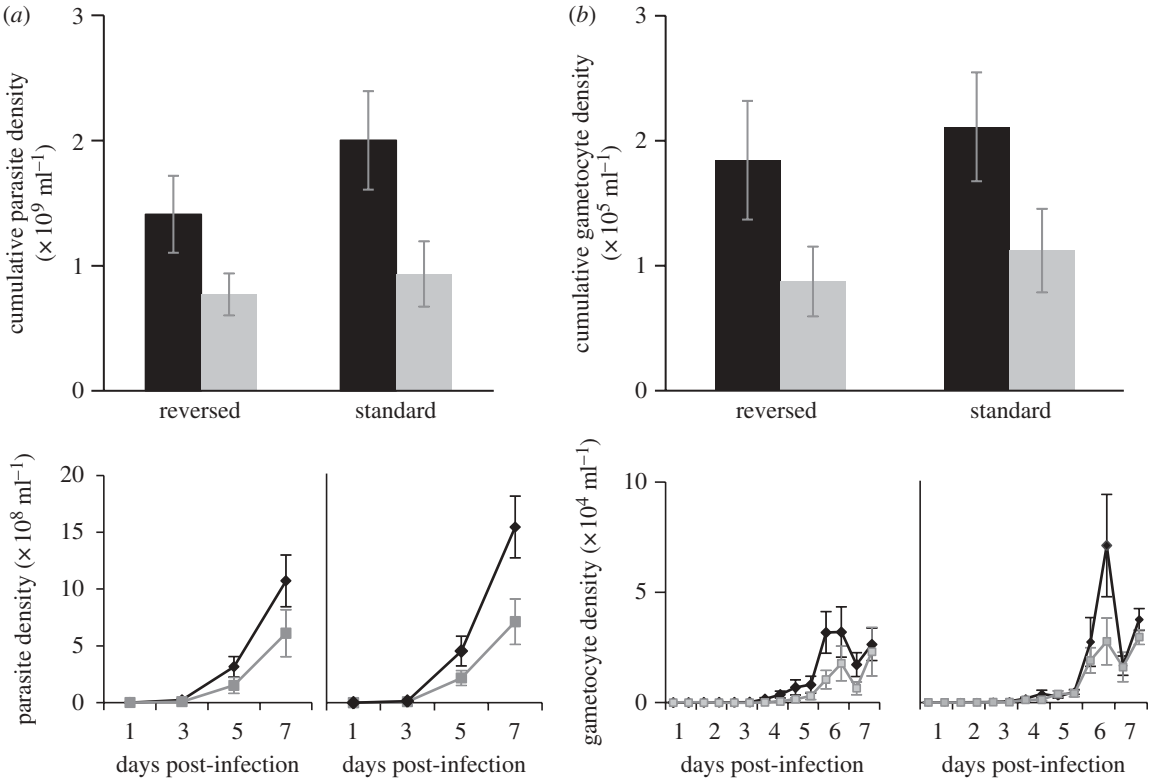

Figure 2. (*a*) Total and (*b*) gametocyte (sexual) densities of infections initiated with parasites matched or mismatched to their host rhythm, in standard and reversed light schedules, followed from (*a*) 24 h or (*b*) 12 h p.i. to 168 h. The top panels show the mean cumulative (±s.e.) densities achieved by infections in hosts ($n = 6$ per group) on the same (matched, black bars) or opposite (mismatched, grey bars) schedule. In each graph, the left pair of bars represents infections originating from the light-reversed room and the right pair of bars represents infections originating from the standard schedule room. The lower panels decompose the cumulative densities into the temporal dynamics of infections in the treatment groups in each room: the mean (±s.e.) densities of matched (black lines) and mismatched (grey lines) infections are plotted. The left graph of each point represents infections originating from the light-reversed room and the right graph of each point represents infections originating from the standard schedule room. The difference in the number of points plotted in the lower panels (and contributing data to the top panels) is due to the lower frequency of analysing samples to count total parasites (*a*) than gametocytes (*b*).

Table 1. The effects of schedule perturbation treatment (matched/mismatched), room (schedule) of origin and their interaction on the performance of infections. *F*-ratios and associated *p*-values for all terms are given along with the mean (±s.e.) difference (matched−mismatched) for significant effects and the adjusted R^2 for minimal models.

	F-ratio	*p*-value	mean difference
parasite density (adj $R^2 = 0.24$)			
treatment (matched/mismatched)	$F_{1,22} = 8.38$	0.008	$8.54 \pm 2.9 \times 10^8$ ml^{-1}
original schedule (reversed/standard)	$F_{1,21} = 0.51$	0.482	—
treatment × schedule	$F_{1,20} = 1.64$	0.215	—
gametocyte density (adj $R^2 = 0.20$)			
treatment (matched/mismatched)	$F_{1,22} = 6.84$	0.016	$9.80 \pm 3.7 \times 10^4$ ml^{-1}
original schedule (reversed/standard)	$F_{1,21} < 0.01$	0.979	—
treatment × schedule	$F_{1,20} = 0.44$	0.516	—
coefficient of variation (adj $R^2 = 0.14$)			
treatment (matched/mismatched)	$F_{1,22} = 4.69$	0.041	0.03 ± 0.015
original schedule (reversed/standard)	$F_{1,21} = 3.35$	0.082	—
treatment × schedule	$F_{1,20} = 1.33$	0.262	—

infections [33,38,39]. Replication rate is also a key factor in determining the production of transmission stages [28]. For the range of gametocyte densities observed in our data, there is a strong positive relationship with mosquito infectivity [28,40–42] in terms of both the prevalence and intensity of mosquitoes infected. The greater variation in cell-cycle schedules in mismatched infections suggests that an interaction between the synchronicity and timing of cell-cycle rhythms shapes the dynamics of infections. More broadly, our data suggest that circadian rhythms play an important role in the evolution of host–parasite interactions. Much recent research on circadian clocks in a disease context focuses on the implications of infection for host rhythms [43–45]. However, our results complement observations that perturbation of host clocks shifts parasites' rhythms—across a variety of taxa

[46–48]—and suggests that rhythms are an important but unappreciated selection pressure on parasites.

Understanding how parasites achieve their coordination will facilitate explaining why parasites synchronize cell cycles with host rhythms. A key question is whether the timing and synchronicity of the cell cycle of parasites is a plastic (actively adjusted) trait. The developmental schedule of an asexual parasite can be conceptually split into remodelling the red blood cell, feeding and, finally, replication. Whether there is plasticity in the duration of these processes is yet to be investigated, but it is possible that development time may trade off against the number of progeny produced since each nucleus within a maturing parasite can divide a different number of times [49]. Recent experiments showing that melatonin can speed up and synchronize the development of *Plasmodium falciparum* in culture suggest that parasites might use host melatonin as a time cue [25]. However, these experiments applied melatonin at substantially higher than physiological concentrations, and we have been unable to repeat these studies in our laboratory (S. E. Reece & H. G. McWatters 2009, unpublished data). If cell-cycle duration is plastic, it will be important to test whether development speeds up or slows down and identify the cues used to schedule development, as this may have implications for disease control. For example, if cell cycles can be slowed, quiescent parasite stages may reduce proliferation rate (reduce pathology), but may also be less sensitive to drug treatment (act as a resistance trait) [36,50–52].

Alternatively, parasite cell-cycle schedules and synchronicity might be passively maintained by host factors with a circadian basis. In this scenario, there may be sufficient variation in the cell-cycle duration of parasites within a cohort that, following perturbation, a proportion will be, by chance, on the correct schedule and so form the next cohort [53]. This could occur as a result of host responses to schizogony, such as fever or cytokine spikes, both of which may kill parasites that are on a slower schedule [54,55]. While fever may play a role in human malaria infections, mice do not experience fever at schizogony, which suggests that other host factors must be involved in synchronizing parasites. Whether synchronicity and circadian development are the results of an active parasite strategy, or a passive host effect, the speed at which parasite schedules recover from perturbation will depend on many factors, including the costs/benefits of mismatch, how much variation exists in the development time of each cohort, the duration of the 'gate' that selects which parasites contribute to the next cell cycle, and the accuracy with which parasites can detect and respond to time cues.

It is important to distinguish between explanations for circadian rhythms that merely require synchronicity and those that require synchronicity to be linked to environmental rhythms. For example, one explanation, discussed above, for parasite synchronicity is that 'safety in numbers' protects progeny when they are released into the blood stream at schizogony. However, this explanation only requires parasites to be coordinated with each other, not with the host. The Hawking hypothesis predicts that parasite cell cycles are timed so that the maturation of each cohort of gametocytes coincides with mosquito-biting activity [56,57], implying that vector rhythms are the relevant environmental parameter. However, data available across a range of *Plasmodium* species are not supportive. For instance, human malaria (*P. falciparum*) gametocytes do not show diurnal rhythms in infectivity to mosquitoes [58,59] and are infectious for at least 7 days [60,61]. Temporal coordination may benefit parasites in two ways: by facilitating exploitation of circadian-dependent host resources, such as the release of new red blood cells [62,63], or avoidance of interactions with host immune factors, such as TNF-α or IL-6, which are secreted with a circadian rhythm [43]. Interestingly, TNF-α is a major component of the immune response (paroxysm) initiated by the synchronous release of parasites at schizogony and can 'sterilize' gametocyte infectivity for several hours [64]. That cell cycles are timed to end at night, when vectors are active, suggests parasite rhythms are either the resolution of a significant trade-off or a serious constraint [14].

Evolutionary ecology is concerned with explaining variation and its fitness consequences. Studies asking the fundamental evolutionary question of why circadian clocks are important for an organism provide the necessary context for work focusing on circadian mechanisms [6]. Circadian clocks have evolved multiple times because many organisms are exposed to the daily changes in light and temperature resulting from the planet's rotation. Across taxa, there is little homology between clock proteins [65], but complex, interlocked feedback loops and close associations with light and/or temperature input pathways are features of all known clockworks [10,66]. The repeated observation of such mechanisms lends support to the idea that the clock's ultimate purpose is to track seasonal changes and couple endogenous timekeeping with environment rhythmicity. Chronobiology has historically been neglected by evolutionary ecologists, but this is changing as it offers a novel opportunity for a holistic approach: because the mechanics of circadian clocks are well known from multiple model systems (including fungi [8,67], insects [7] and mammals [9]), there are real opportunities to link mechanistic and evolutionary explanations for an important trait. However, there are substantial challenges associated with linking trait variation and underlying physiological mechanisms, not least the difficulty of assessing the effect of the clock on fitness in a context resembling that of the real world (most experimental designs have considered the effects of non-24 h light : dark cycles [3–5]). The wealth of cell and molecular biology data available for malaria parasites, and the ability to investigate and manipulate their traits *in vivo* and *in vitro*, offer a powerful means to set chronobiology within an evolutionary framework.

All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

We thank J. Thompson and N. Savill for discussion, L. Carter, M. Sontheim and G. K. P. Barra for assistance, four anonymous reviewers for help in improving the manuscript, the Wellcome Trust (A.J.O.D., P.S., S.R.) and the Royal Society (H.G.M.) for funding.

REFERENCES

1 Dunlap, J. C., Loros, J. J. & Decoursey, P. J. 2004 *Chronobiology: biological timekeeping.* Sunderland, UK: Sinauer Associates.

2 Pittendrigh, C. S. 1960 Circadian rhythms and the circadian organization of living systems. In *Cold Spring Harbor symposium on quantitative biology*, pp. 159–184. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

3 Ouyang, Y., Andersson, C. R., Kondo, T., Golden, S. S. & Johnson, C. H. 1998 Resonating circadian clocks enhance fitness in cyanobacteria. *Proc. Natl Acad. Sci. USA* **95**, 8660–8664. (doi:10.1073/pnas.95.15.8660)

4 Dodd, A. N., Salathia, N., Hall, A., Kevei, E., Toth, R., Nagy, F., Hibberd, J. M., Millar, A. J. & Webb, A. A. R. 2005 Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. *Science* **309**, 630–633. (doi:10.1126/science.1115581)

5 Saunders, D. S. 1972 Circadian control of larval growth rate in *Sarcophaga argyrostoma*. *Proc. Natl Acad. Sci. USA* **69**, 2738–2740. (doi:10.1073/pnas.69.9.2738)

6 Yerushalmi, S. & Green, R. M. 2009 Evidence for the adaptive significance of circadian rhythms. *Ecol. Lett.* **12**, 970–981. (doi:10.1111/j.1461-0248.2009.01343.x)

7 Allada, R. & Chung, B. Y. 2010 Circadian organization of behavior and physiology in *Drosophila*. *Annu. Rev. Physiol.* **72**, 605–624. (doi:10.1146/annurev-physiol-021909-135815)

8 Brunner, M. & Kaldi, K. 2008 Interlocked feedback loops of the circadian clock of *Neurospora crassa*. *Mol. Microbiol.* **68**, 255–262. (doi:10.1111/j.1365-2958.2008.06148.x)

9 Dibner, C., Schibler, U. & Albrecht, U. 2010 The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu. Rev. Physiol.* **72**, 517–549. (doi:10.1146/annurev-physiol-021909-135821)

10 Stelling, J., Gilles, E. D. & Doyle, F. J. 2004 Robustness properties of circadian clock architectures. *Proc. Natl Acad. Sci. USA* **101**, 13 210–13 215. (doi:10.1073/pnas.0401463101)

11 Troein, C., Locke, J. C. W., Turner, M. S. & Millar, A. J. 2009 Weather and seasons together demand complex biological clocks. *Curr. Biol.* **19**, 1961–1964. (doi:10.1016/j.cub.2009.09.024)

12 DeCoursey, P. J., Walker, J. K. & Smith, S. A. 2000 A circadian pacemaker in free-living chipmunks: essential for survival? *J. Comp. Physiol. A* **186**, 169–180. (doi:10.1007/s003590050017)

13 Hawking, F. 1970 The clock of the malaria parasite. *Sci. Am.* **222**, 123–131. (doi:10.1038/scientificamerican0670-123)

14 Reece, S. E., Ramiro, R. S. & Nussey, D. H. 2009 Plastic parasites: sophisticated strategies for survival and reproduction? *Evol. Appl.* **2**, 11–23. (doi:10.1111/j.1752-4571.2008.00060.x)

15 Babiker, H. A., Schneider, P. & Reece, S. E. 2008 Gametocytes: insights gained during a decade of molecular monitoring. *Trends Parasitol.* **24**, 525–530. (doi:10.1016/j.pt.2008.08.001)

16 Cheesman, S. J., de Roode, J. C., Read, A. F. & Carter, R. 2003 Real-time quantitative PCR for analysis of genetically mixed infections of malaria parasites: technique validation and applications. *Mol. Biochem. Parasitol.* **131**, 83–91. (doi:10.1016/S0166-6851(03)00195-6)

17 Wargo, A. R., Randle, N., Chan, B. H. K., Thompson, J., Read, A. F. & Babiker, H. A. 2006 *Plasmodium chabaudi*: reverse transcription PCR for the detection and quantification of transmission stage malaria parasites. *Exp. Parasitol.* **112**, 13–20. (doi:10.1016/j.exppara.2005.08.013)

18 Foster, K. R. 2005 Biomedicine—Hamiltonian medicine: why the social lives of pathogens matter. *Science* **308**, 1269–1270. (doi:10.1126/science.1108158)

19 Paul, R. E. L., Ariey, F. & Robert, V. 2003 The evolutionary ecology of *Plasmodium*. *Ecol. Lett.* **6**, 866–880. (doi:10.1046/j.1461-0248.2003.00509.x)

20 Stearns, S. C. & Koella, J. C. 2008 *Evolution in health and disease*. Oxford, UK: Oxford University Press.

21 Gautret, P., Deharo, E., Tahar, R., Chabaud, A. G. & Landau, I. 1995 The adjustment of the schizogonic cycle of *Plasmodium chabaudi chabaudi* in the blood to the circadian rhythm of the host. *Parasite* (*Paris, France*) **2**, 69–74.

22 Taliaferro, L. G. 1928 Return to normal of the asexual cycle in bird malaria after retardation by low temperatures *in vitro*. *J. Prev. Med.* **2**, 525–541.

23 Taliaferro, W. H. & Taliaferro, L. G. 1934 Alteration in the time of sporulation of *Plasmodium brasilianum* in monkeys by reversal of light and dark. *Am. J. Epidemiol.* **20**, 50–59.

24 Trager, W. & Jensen, J. B. 1976 Human malaria parasites in continuous culture. *Science* **193**, 673–675. (doi:10.1126/science.781840)

25 Hotta, C. T., Gazarini, M. L., Beraldo, F. H., Varotti, F. P., Lopes, C., Markus, R. P., Pozzan, T. & Garcia, C. R. S. 2000 Calcium-dependent modulation by melatonin of the circadian rhythm in malarial parasites. *Nat. Cell Biol.* **2**, 466–468. (doi:10.1038/35017112)

26 Killick-Kendrick, R. & Peters, W. 1978 *Rodent malaria*. New York, NY: Academic Press.

27 Vitaterna, M. H. *et al.* 1994 Mutagenesis and mapping of a mouse gene clock, essential for circadian behavior. *Science* **264**, 719–725. (doi:10.1126/science.8171325)

28 Mackinnon, M. J. & Read, A. F. 1999 Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*. *Evolution* **53**, 689–703. (doi:10.2307/2640710)

29 Wargo, A. R., de Roode, J. C., Huijben, S., Drew, D. R. & Read, A. F. 2007 Transmission stage investment of malaria parasites in response to in-host competition. *Proc. R. Soc. B* **274**, 2629–2638. (doi:10.1098/rspb.2007.0873)

30 Garnham, P. C. C. 1966 *Malaria parasites and other haemosporidia*. Oxford, UK: Blackwell Scientific.

31 Mota, M. M., Jarra, W., Hirst, E., Patnaik, P. K. & Holder, A. A. 2000 *Plasmodium chabaudi*-infected erythrocytes adhere to CD36 and bind to microvascular endothelial cells in an organ-specific way. *Infect. Immun.* **68**, 4135–4144. (doi:10.1128/IAI.68.7.4135-4144.2000)

32 Crawley, M. J. 2007 *The R book*. Chichester, UK: John Wiley & Sons Ltd.

33 Bell, A. S., de Roode, J. C., Sim, D. & Read, A. F. 2006 Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. *Evolution* **60**, 1358–1371.

34 Mackinnon, M. J. & Read, A. F. 2004 Immunity promotes virulence evolution in a malaria model. *PLoS Biol.* **2**, 1286–1292. (doi:10.1371/journal.pbio.0020230)

35 Reece, S. E., Ali, E., Schneider, P. & Babiker, H. A. 2010 Stress, drugs and the evolution of reproductive restraint in malaria parasites. *Proc. R. Soc. B* **277**, 3123–3129. (doi:10.1098/rspb.2010.0564)

36 Schneider, P., Chan, B. H. K., Reece, S. E. & Read, A. F. 2008 Does the drug sensitivity of malaria parasites depend on their virulence? *Malar. J.* **7**, 257. (doi:10.1186/1475-2875-7-257)

37 Pollitt, L. C., Mideo, N., Drew, D. R., Colegrave, N. & Reece, S. E. In press. Coping with competition: strategies for sex and survival. *Am. Nat.*

38 de Roode, J. C., Helinski, M. E., Anwar, M. A. & Read, A. F. 2005 Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *Am. Nat.* **166**, 531–542. (doi:10.1086/491659)

39 de Roode, J. C., Read, A., Chan, B. & Mackinnon, M. J. 2003 Rodent malaria parasites suffer from the presence of conspecific clones in three-clone *Plasmodium chabaudi* infections. *Parasitology* **127**, 411–418. (doi:10.1017/S0031182003004001)

40 Drakeley, C., Sutherland, C., Bousema, J. T., Sauerwein, R. W. & Targett, G. A. T. 2006 The epidemiology of *Plasmodium falciparum* gametocytes: weapons of mass dispersion. *Trends Parasitol.* **22**, 424–430. (doi:10.1016/j.pt.2006.07.001)

41 Mackinnon, M. J. & Read, A. F. 2004 Virulence in malaria: an evolutionary viewpoint. *Phil. Trans. R. Soc. Lond. B* **359**, 965–986. (doi:10.1098/rstb.2003.1414)

42 Schneider, P., Bousema, J., Gouagna, L. C., Otieno, S., Van De Vegte-Bolmer, M., Omar, S. A. & Sauerwein, R. W. 2007 Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. *Am. J. Trop. Med. Hyg.* **76**, 470–474.

43 Keller, M., Mazuch, J., Abraham, U., Eom, G. D., Herzog, E. D., Volk, H. D., Kramer, A. & Maier, B. 2009 A circadian clock in macrophages controls inflammatory immune responses. *Proc. Natl Acad. Sci. USA* **106**, 21 407–21 412. (doi:10.1073/pnas.0906361106)

44 Peng, Z. C., Kristensson, K. & Bentivoglio, M. 1994 Dysregulation of photic induction of Fos-related protein in the biological clock during experimental trypanosomiasis. *Neurosci. Lett.* **182**, 104–106. (doi:10.1016/0304-3940(94)90217-8)

45 Sanchez, Y., Rosado, J. D., Vega, L., Elizondo, G., Estrada-Muniz, E., Saavedra, R., Juarez, I. & Rodriguez-Sosa, M. 2010 The unexpected role for the aryl hydrocarbon receptor on susceptibility to experimental toxoplasmosis. *J. Biomed. Biotechnol.* **2010**, 505694. (doi:10.1155/2010/505694)

46 Cornford, E. M., Freeman, B. J. & MacInnis, A. J. 1976 Physiological relationships and circadian periodicities in rodent trypanosomes. *Trans. R. Soc. Trop. Med. Hyg.* **70**, 238–243. (doi:10.1016/0035-9203(76)90047-X)

47 Fantinou, A. A., Alexandri, M. P. & Tsitsipis, J. A. 1998 Adult emergence rhythm of the egg-parasitoid *Telenomus busseolae*. *Biocontrol* **43**, 141–151. (doi:10.1023/A:1009984318289)

48 Toledo, R., Muñoz-Antoli, C. & Esteban, J. G. 1999 Production and chronobiology of emergence of the cercariae of *Euparyphium albuferensls* (Trematoda: Echinostomatidae). *J. Parasitol.* **85**, 263–267. (doi:10.2307/3285630)

49 Leete, T. H. & Rubin, H. 1996 Malaria and the cell cycle. *Trends Parasitol.* **12**, 442–444.

50 Teuscher, F., Gatton, M. L., Chen, N., Peters, J., Kyle, D. E. & Cheng, Q. 2010 Artemisinin-induced dormancy in *Plasmodium falciparum*: duration, recovery rates, and implications in treatment failure. *J. Infect. Dis.* **202**, 1362–1368. (doi:10.1086/656476)

51 Veiga, M. I., Ferreira, P. E., Schmidt, B. A., Ribacke, U., Bjorkman, A., Tichopad, A. & Gil, J. P. 2010 Antimalarial exposure delays *Plasmodium falciparum* intra-erythrocytic cycle and drives drug transporter genes expression. *PLoS ONE* **5**. (doi:10.1371/journal.pone.0012408)

52 Witkowski, B., Lelievre, J., Barragan, M. J., Laurent, V., Su, X. Z., Berry, A. & Benoit-Vical, F. 2010 Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. *Antimicrob. Agents Chemother.* **54**, 1872–1877. (doi:10.1128/AAC.01636-09)

53 Rouzine, I. M. & McKenzie, F. E. 2003 Link between immune response and parasite synchronization in malaria. *Proc. Natl Acad. Sci. USA* **100**, 3473–3478. (doi:10.1073/pnas.262796299)

54 Gravenor, M. B. & Kwiatkowski, D. 1998 An analysis of the temperature effects of fever on the intra-host population dynamics of *Plasmodium falciparum*. *Parasitology* **117**, 97–105. (doi:10.1017/S0031182098002893)

55 Kwiatkowski, D. 1989 Febrile temperatures can synchronize the growth of *Plasmodium falciparum in vitro*. *J. Exp. Med.* **169**, 357–361. (doi:10.1084/jem.169.1.357)

56 Garnham, P. C. C. & Powers, K. G. 1974 Periodicity of infectivity of plasmodial gametocytes: the Hawking phenomenon. *Int. J. Parasitol.* **4**, 103–106. (doi:10.1016/0020-7519(74)90014-9)

57 Hawking, F., Worms, M. J. & Gammage, K. 1968 24- and 48-hour cycles of malaria parasites in blood—their purpose production and control. *Trans. R. Soc. Trop. Med. Hyg.* **62**, 731–760. (doi:10.1016/0035-9203(68)90001-1)

58 Bray, R. S., McCrae, A. W. R. & Smalley, M. E. 1976 Lack of a circadian rhythm in the ability of the gametocytes of *Plasmodium falciparum* to infect *Anopheles gambiae*. *Int. J. Parasitol.* **6**, 399–401. (doi:10.1016/0020-7519(76)90025-4)

59 Githeko, A. K., Brandling-Bennett, A. D., Beier, M., Mbogo, C. M., Atieli, F. K., Owaga, M. L., Juma, F. & Collins, F. H. 2008 Confirmation that *Plasmodium falciparum* has aperiodic infectivity to *Anopheles gambiae*. *Med. Vet. Entomol.* **7**, 373–376. (doi:10.1111/j.1365-2915.1993.tb00708.x)

60 Magesa, S. M., Mdira, Y. K., Akida, J. A., Bygbjerg, I. C. & Jakobsen, P. H. 2000 Observations on the periodicity of *Plasmodium falciparum* gametocytes in natural human infections. *Acta Trop.* **76**, 239–246. (doi:10.1016/S0001-706X(00)00110-8)

61 Smalley, M. E. & Sinden, R. E. 2009 *Plasmodium falciparum* gametocytes: their longevity and infectivity. *Parasitology* **74**, 1–8. (doi:10.1017/S0031182000047478)

62 Clark, R. H. & Korst, D. R. 1969 Circadian periodicity of bone marrow mitotic activity and reticulocyte counts in rats and mice. *Science* **166**, 236–237. (doi:10.1126/science.166.3902.236)

63 Savill, N. J., Chadwick, W. & Reece, S. E. 2009 Quantitative analysis of mechanisms that govern red blood cell age structure and dynamics during anaemia. *PLoS Comput. Biol.* **5**, e1000416. (doi:10.1371/journal.pcbi.1000416)

64 Naotunne, T. D., Karunaweera, N. D., Delgiudice, G., Kularatne, M. U., Grau, G. E., Carter, R. & Mendis, K. N. 1991 Cytokines kill malaria parasites during infection crisis—extracellular complementary factors are essential. *J. Exp. Med.* **173**, 523–529. (doi:10.1084/jem.173.3.523)

65 Rosbash, M. 2009 The implications of multiple circadian clock origins. *PLoS Biol.* **7**, 421–425. (doi:10.1371/journal.pbio.1000062)

66 Rand, D. A., Shulgin, B. V., Salazar, D. & Millar, A. J. 2004 Design principles underlying circadian clocks. *J. R. Soc. Interface* **1**, 119–130. (doi:10.1098/rsif.2004.0014)

67 Merrow, M. & Roenneberg, T. 2007 Circadian entrainment of *Neurospora crassa*. *Clocks Rhythms* **72**, 279–285.

Paper #2 (2013 & 2014): Disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated

MALARIA JOURNAL

RESEARCH Open Access

Disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated

Aidan J O'Donnell[1*], Nicole Mideo[2] and Sarah E Reece[1,3]

Abstract

Background: In the blood, the synchronous malaria parasite, *Plasmodium chabaudi*, exhibits a cell-cycle rhythm of approximately 24 hours in which transitions between developmental stages occur at particular times of day in the rodent host. Previous experiments reveal that when the timing of the parasite's cell-cycle rhythm is perturbed relative to the circadian rhythm of the host, parasites suffer a (~50%) reduction in asexual stages and gametocytes. Why it matters for parasites to have developmental schedules in synchronization with the host's rhythm is unknown. The experiment presented here investigates this issue by: (a) validating that the performance of *P. chabaudi* is negatively affected by mismatch to the host circadian rhythm; (b) testing whether the effect of mismatch depends on the route of infection or the developmental stage of inoculated parasites; and, (c) examining whether the costs of mismatch are due to challenges encountered upon initial infection and/or due to ongoing circadian host processes operating during infection.

Methods: The experiment simultaneously perturbed the time of day infections were initiated, the stage of parasite inoculated, and the route of infection. The performance of parasites during the growth phase of infections was compared across the cross-factored treatment groups (i e, all combinations of treatments were represented).

Results: The data show that mismatch to host rhythms is costly for parasites, reveal that this phenomenon does not depend on the developmental stage of parasites nor the route of infection, and suggest that processes operating at the initial stages of infection are responsible for the costs of mismatch. Furthermore, mismatched parasites are less virulent, in that they cause less anaemia to their hosts.

Conclusion: It is beneficial for parasites to be in synchronization with their host's rhythm, regardless of the route of infection or the parasite stage inoculated. Given that arrested cell-cycle development (quiescence) is implicated in tolerance to drugs, understanding how parasite schedules are established and maintained in the blood is important.

Keywords: Developmental rhythms, Circadian clock, Fitness, Malaria, Ring stage, Trophozoite, Intravenous, Intraperitoneal, Synchronicity, Phase-shift

Background

Biological rhythms are ubiquitous in taxa spanning bacteria to vertebrates, eliciting periodicity in a multitude of biological processes and behaviours. Accurately matching biological rhythms to the daily rotation of the Earth appears to be important for competitive ability (cyanobacteria and plants) [1,2], growth rate (insects) [3], and reproductive success (plants and insects) [4-7]. In the blood, the synchronous malaria parasite, *Plasmodium chabaudi*, exhibits a cell-cycle rhythm of approximately 24 hours in which transitions between developmental stages occur at particular times of day in the rodent host (Figure 1) [8]. Such synchronous development has been documented in many species of malaria parasite, including those that infect humans (reviewed in [9]). Perturbing the timing of the *P. chabaudi* cell cycle relative to the host's circadian rhythm causes a two-fold reduction in the densities of both asexual and sexual transmission stages [10]. This has implications for parasite fitness because low densities of asexual stages make parasites vulnerable to clearance by

* Correspondence: Aidan.Odonnell@ed.ac.uk
[1]Institutes of Evolution, Immunology and Infection Research, University of Edinburgh, Edinburgh, UK
Full list of author information is available at the end of the article

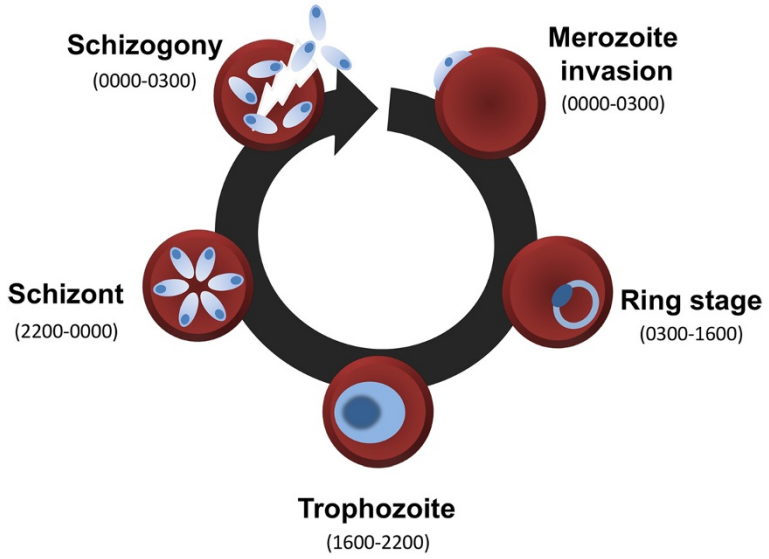

Figure 1 The cell cycle of *Plasmodium*. For *Plasmodium chabaudi*, progressing through these developmental stages takes 24 hours. Approximate host circadian times are given in parentheses.

the immune system and poor competitors in mixed infections and (in general) sexual stage density correlates positively with the success of transmission to mosquitoes [11-15]. While the net fitness costs for parasites of perturbing their coordination with the biological rhythm of the host are apparent, the processes that reduce asexual and gametocyte densities during perturbation are unknown.

The reduced performance of schedule mismatched parasites observed in [10] does not reveal whether coordination between parasite cell-cycle progression and the host circadian rhythm is controlled by parasites or hosts or both. This remains an important route of future investigation which will be facilitated by better characterisation of the costs of mismatch. This includes determining when the costs of mismatch materialize: are the costs of mismatch a result of time-of-day-dependent challenges encountered upon initial infection and/or challenges experienced continuously throughout infections? Though the cell cycles of mismatched parasites eventually adjust to be in synchrony with the host circadian rhythm [16], prior to this, parasites in each cell cycle may enter a particularly vulnerable stage in their development at a time when circadian aspects of the within-host environment are least favourable. For example, parasite developmental stages may vary in their sensitivity to peaks in the rhythms of innate immune defences in the blood/spleen or the nutritional requirements of different stages may not be met at certain times of day. These time-of-day dependent challenges could affect parasites as they enter the host (if, for instance, low densities of parasites are particularly vulnerable, or these processes operate at the site of infection) and/or during every cycle as infections progress. Distinguishing between these alternatives is non-trivial, not least because even small costs that arise during initial establishment will propagate and magnify with successive rounds of replication, resulting in reduced overall performance. However, a clear prediction is that if mismatch causes costs in the initial phase of infections there will be fewer parasites appearing in the blood and if costs are due to ongoing processes, there will be differences in multiplication rate throughout infections.

This study asks when the costs of mismatch appear and also addresses two issues raised by the results of [10]. First, the route of infection in [10] was via intraperitoneal injection, either in the host's morning or evening. If circadian host processes play a role in the establishment phase of experimental infections, then mismatched parasites may have performed poorly because of time-of-day dependent challenges experienced in the peritoneal cavity. For example, given the circadian periodicity of macrophage activity [17], parasites injected in the evening were likely to encounter peritoneal macrophages in the peak of their protective activity. In this case, the costs of mismatch would arise in the initial stage of infections, but since the peritoneal cavity is not the natural mode of infection, nor an environment blood stage malaria parasites naturally encounter, the effects reported in [10] may not be biologically relevant. Second, the same parasite stage (rings) was used to establish the infections in [10], but parasite cell-cycle stages may differ in their sensitivity to time-of-day-dependent challenges. For example, different stages may be more sensitive to peritoneal macrophages at the peak of their activity. In this case, the costs of mismatch may be due to an interaction between host time of day and the parasite developmental stage

injected. Characterising how the of costs of mismatch are affected by timing, route of infection, and parasite developmental stage will help to identify the mechanisms underpinning parasite schedules and could provide new insight for control. For example, drugs given at certain times of day could be more effective through synergy with host circadian immune responses or by targeting parasites at their most vulnerable cell-cycle stage.

The aims of the experiment reported here were to validate that the performance of *P. chabaudi* is negatively affected by mismatch to the host circadian rhythm, test whether the costs of mismatch are influenced by the route of infection or the developmental stage of inoculated parasites, and to examine whether the costs of mismatch are due to challenges encountered upon initial infection or to processes operating throughout the infection. This required simultaneously perturbing the stage of parasite inoculated, host time of day, and route of infection, and measuring parasite performance at the start and during infections. The impact to the host is also considered, using red blood cell loss as a measure of parasite virulence [11,18,19]. The results confirm that mismatch to host rhythms is costly for parasites, reveal that this phenomena does not depend on the developmental stage of parasites nor the route of infection (i e, it is not simply a consequence of challenges experienced in the peritoneal cavity), and suggest that processes operating at the initial stages of infection are responsible for the costs of mismatch.

Methods

Parasites and hosts

Hosts were ten to 12-week old MF1 male mice housed at 21°C with *ad lib* food and drinking water supplemented with 0.05% para-aminobenzoic acid (to support parasite growth). The synchronous *P. chabaudi* clone (AJ) was used [10]. Manipulating the circadian rhythms of hosts was achieved by housing mice in two rooms, each maintained on a 12-hour light: dark cycle that differed only in the timing of lights-on. In the "standard schedule" room, lights were on during the day (lights on: 07.30; lights off: 19.30); in the "light reversed" room, lights were on during the night (lights on 19.30; lights off: 07.30). All mice in the experiment were allowed to acclimatize to their respective light: dark schedule for two weeks before infection. This allowed mice to entrain to their schedule, as previous work has demonstrated this occurs within seven days [20]. Prior to infection it was verified that the mice behaved as expected for their light: dark schedule (e.g., were active during the dark period and inactive when lights were on). In each room, a donor host was infected with 1×10^6 *P. chabaudi* (clone AJ) parasitized red blood cells (RBCs) to provide parasites to initiate experimental infections. All procedures were carried out in accordance with the UK Home Office regulations (Animals Scientific Procedures Act 1986; 60/4121) and approved by the ethical review panel at Edinburgh University.

Experimental design

Mice for the experiment were housed in groups of five and a total of 40 were used (n = 5 infections per treatment group). All experimental infections were initiated in the morning (11.00 GMT) (Figure 2). This permitted simultaneous infections using two different parasite stages. Donor infections originating from the "standard schedule" room were used to *simultaneously* infect mice in the "standard schedule" room and the "light reversed room" with ring-stage parasites (hereafter, rings). The same procedure was repeated for parasites from the "light-reversed" room to *simultaneously* initiate infections in each room with late trophozoite-stage parasites (hereafter, trophozoites). This created two groups of infections in which parasite stage and host circadian rhythm were *matched* (e.g., mice in their morning received rings, and mice in their evening received trophozoites) and *mismatched* (e.g., mice in their morning received trophozoites, and mice in their evening received rings). That parasites were at the required stage for initiating infections was verified via blood smear at the time of harvesting. Parasites were administered either via intraperitoneal injection (IP) or intravenous injection (IV), at a dose of 1×10^6 parasitized RBC. This created a total of eight treatments (Figure 2) to include all combinations (cross-factoring) of route of infection (IP or IV), parasite stage (ring or trophozoite), and parasite and host rhythms (schedule matched or mismatched).

Data collection

All mice were sampled daily, in the morning at 09.00 GMT (e.g., beginning at 22 hours post infection), during the growth phase of *P. chabaudi* AJ infections (until day 7 post infection (pi) when starting with 10^6 parasitized RBC [21]). This timing is consistent with previous work [10] and is prior to any adjustment of the schedule of mismatched parasites to become synchronised with the host rhythm [10,16,22-24]. At each sampling point, 5 μl blood samples were taken to quantify total parasite densities using quantitative PCR (qPCR). DNA was extracted using the ABI Prism 6100® according to the manufacturer's protocol. Total parasite densities were obtained using primers based on the gametocyte-expressed gene PC302249.00.0 [25]. RBC densities were measured on days 1, 3 and 7 pi using flow cytometry (Beckman Coulter).

Data analysis

R version 2.6.1 [26] was used for all analyses. General linear models were used to test how the perturbations of the

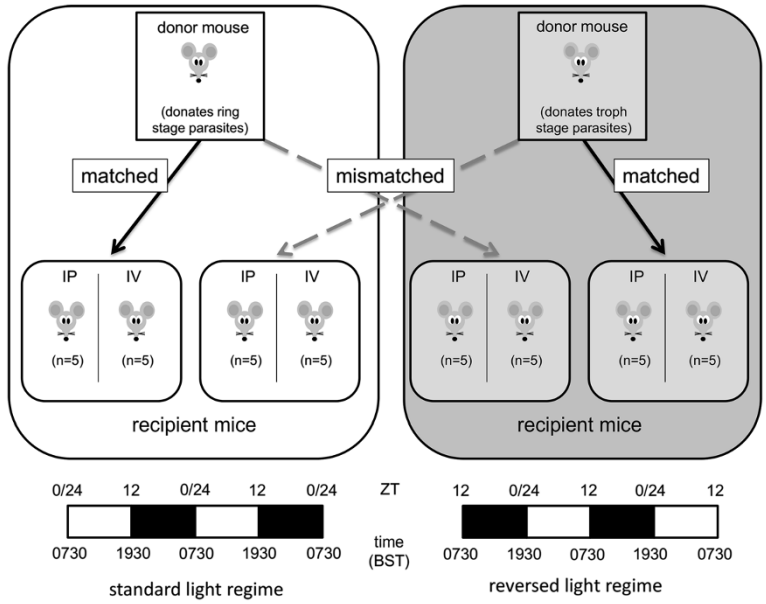

Figure 2 Experimental design. Arrows indicate the transfer of parasites to recipient hosts (eight groups of five mice) within and between two rooms with opposite light schedules. Parasites remaining in the same room are matched to host rhythms and act as controls. Parasites transferred between rooms to hosts that are on the opposite rhythm to the parasite donor were temporally mismatched, analogous to jetlag. At the time of transfer, parasites originating from the standard light regime donor were at ring stage and those originating from the reversed light regime donor were at trophozoite stage and infections were initiated via either intraperitoneal (IP) or intravenous (IV) routes. Dark and light bars indicate lights on/off status throughout a 48-hour period. Zeitgeber time (ZT) is displayed above the bars; ZT 0/24, the time of lights on and ZT 12, time of lights off.

route of infection, parasite stage, and co-ordination of parasite and host rhythms affected (i) the ability of parasites to establish infections (days 1 and 2 pi) and, (ii) their overall performance to the peak of infections (cumulative density between days 1–7). General linear mixed effects models were used to examine whether replication rate was affected by mismatch of host and parasite rhythms. This required fitting mouse identity as random effect to control for the non-independence of multiple data points from each infection [27]. Maximal models contained all main effects and interactions, and models were minimized using stepwise deletion until only significant terms remained. Parasite multiplication rate was calculated as the number of parasites observed on day $t + 1$ divided by the number on the previous day (t).

Results

The route of infection, parasite stage, and mismatch between host and parasite schedules all had significant effects on parasite densities (Figure 3). Infections via IV had significantly higher densities on day 1 ($F_{(1,\ 36)} = 14.70$; $P < 0.001$) and 2 ($F_{(1,\ 36)} = 15.50$; $P < 0.001$) pi, and this was maintained throughout the pre-peak phase of the infection (as demonstrated by cumulative parasites densities; $F_{(1,\ 36)} = 10.09$; $P = 0.003$). Infections initiated with rings performed significantly better than infections initiated with trophozoites on day 1 ($F_{(1,\ 36)} = 12.75$; $P = 0.001$) and 2 ($F_{(1,\ 36)} = 16.10$; $P < 0.001$) pi, and throughout the pre-peak phase of the infection (cumulative parasites densities; $F_{(1,\ 36)} = 15.89$; $P < 0.001$). On day 1 post-infection, the densities of matched and mismatched parasite densities did not differ significantly ($F_{(1,\ 36)} = 1.76$; $P = 0.193$) though the densities of mismatched parasites tended to be lower. By day 2, however, matched parasites performed significantly better than mismatched parasites ($F_{(1,\ 36)} = 4.33$; $P = 0.045$) and this pattern was maintained throughout the pre-peak phase (cumulative parasites densities; $F_{(1,\ 36)} = 26.01$; $P < 0.001$), as can be seen in the temporal dynamics (Figure 4). The means (±se) for the significant effects and R squared values for the minimal models are given in Table 1.

There were no significant interactions between host-parasite schedules and the route of infection or parasite stage (all $P > 0.60$). This reveals that mismatch has equal effects on parasites administered IP and IV, and on ring and trophozoite stages. This allows treatment groups to be combined to directly compare matched with mismatched parasites to examine whether the costs of mismatch stem from processes that operate during infections to constrain replication (Figure 5). The number of progeny produced by each parasite (multiplication rate) varies during infections ($\chi^2_5 = 263.31$; $P < 0.001$) but does not differ significantly

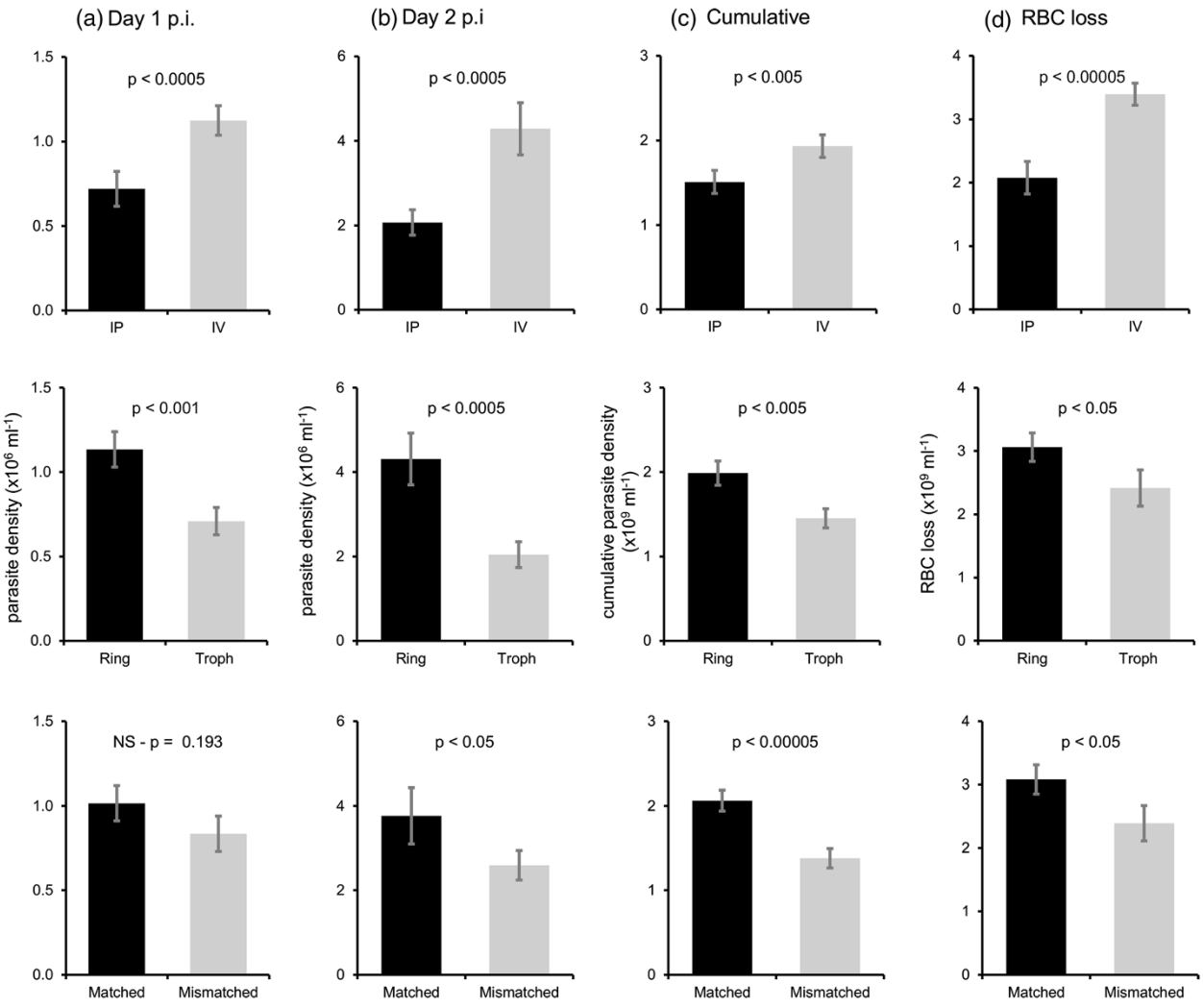

Figure 3 Parasites performed better when injected via intravenous, at ring-stage, and matched to host rhythms, resulting in greater virulence. Total parasite densities of infections **(a)** on day 1 post-infection, **(b)** on day 2 post infection, and **(c)** summed from day 1 to day 7 pi (cumulative density); **(d)** overall loss of RBCs during the pre-peak phase. Bars show mean (±se) densities of parasites or RBC with (a) n = 39 infections per group (b-d) n = 40 per group. The top row compares the route of infection either by IP (intraperitoneal injection, black bars) or IV (intravenous injection, grey bars). The middle row compares the parasite stage used to initiate the infections, with rings (black bars) or trophozoites (grey bars). The bottom row compares parasites on the same (matched, black bars) or perturbed (mismatched, grey bars) schedule as the host.

between matched and mismatched parasites, for all cell cycles examined (Schedule: $\chi^2_1 = 0.01$; P = 0.964; day by schedule interaction: $\chi^2_5 = 1.86$; P = 0.868). This result, taken together with the significant difference in densities appearing by day 2 pi suggests that circadian processes operating in the initial phase of infection reduce parasite number and this initial difference is propagated throughout infections to result in significant costs of mismatch with the host rhythm.

It is easy to show algebraically that any small difference in initial parasite densities between matched and mismatched parasites will increase at a rate proportional to the multiplication rate, even when each parasite produces the same number of progeny per cell cycle. If the initial densities of matched and mismatched parasites are p and $p + \varepsilon$, respectively, and the multiplication rate of all parasites is r, then after t days (rounds of replication) the density of matched and mismatched parasites will be $r^t p$ and $r^t (p + \varepsilon)$ and the difference in densities between matched and mismatched infections will have increased by a factor of r^t (i e, from ε to $r^t \varepsilon$). Even if multiplication rates change over time (i e, r changes over time, as is the case; Figure 5), as long as it is greater than 1, the difference between matched and mismatched parasite densities will increase as infections progress.

Finally, hosts lost RBCs throughout the pre-peak phase of the infection and the patterns mirrored parasite performance. Hosts infected via IV lost significantly more

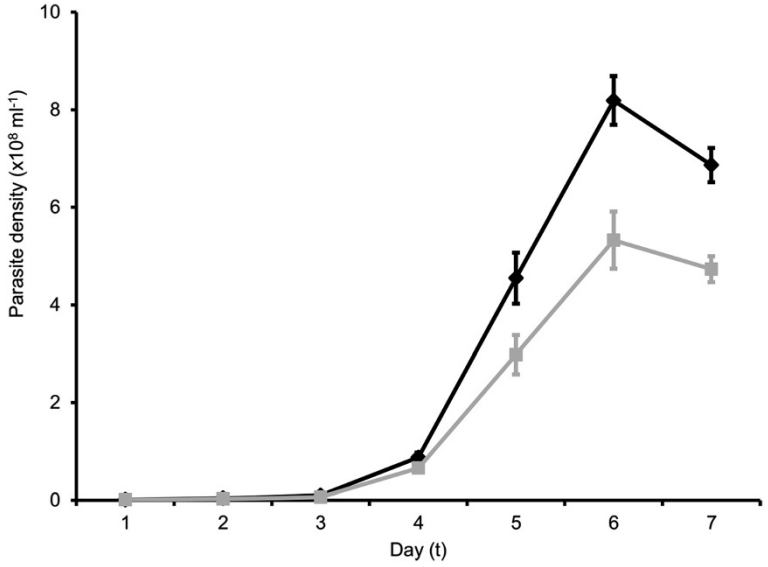

Figure 4 Matched parasites performed better than mismatched parasites throughout the pre-peak phase of the infection. Temporal dynamics of all infections (route and stage treatments combined) followed from day 1 to day 7 pi. The mean (±se) densities of matched (black lines) and mistmatched (grey lines) infections are plotted.

RBC (i e, had greater anaemia) than via IP ($F_{(1, 36)} = 22.49$; $P < 0.001$), hosts receiving ring-stage parasites lost more RBCs than those receiving trophozoites ($F_{(1, 36)} = 5.36$; $P = 0.026$), and matched parasites caused greater anaemia than mismatched parasites ($F_{(1, 36)} = 6.13$; $P = 0.018$). Again there were no significant interactions (all $P > 0.29$) between schedule, route, and stage affecting RBC loss.

Discussion

This experiment involved the simultaneous perturbation of coordination between host and parasite schedules, the stage of parasite inoculated, and the route of infection. The data confirm that mismatch to host rhythms is costly for *P. chabaudi* parasites and reveal that this phenomena does not depend on the developmental stage inoculated nor the route of infection. Coupled with previous work [10], the data demonstrate that a phase-shift of between nine to 12 hours is detrimental for parasites. Moreover, further analyses reject the hypothesis that the costs of mismatch are due to processes that reduce the multiplication rate of parasites throughout infections, but instead, suggest that processes operating when parasites are establishing a blood stage infection are responsible. The lack of impact of time-of-day effects throughout infections cannot be explained by parasite schedules quickly adjusting to become synchronised with the host circadian rhythm. By staging parasites in blood smears we verified that, 3 days after inoculation, parasites were maintaining their original developmental schedule (data not shown), and previous work suggests that any adjustment takes at least 7 days [10,16,22-24].

The experiment also revealed that, as expected, ring stage parasites are more successful in establishing infections (which is presumably why, conventionally, ring stages initiate experimental infections) than trophozoite stages and both stages benefit from being injected straight into the blood stream rather than having to negotiate their way from the peritoneal cavity to the blood (by an as yet unknown mechanism). The effects of parasite stage and route of infection were apparent by 1 pi. Finally, the negative effects of schedule mismatch on parasite performance have consequences for virulence because hosts receiving mismatched parasites suffer less anaemia than those infected with matched parasites.

Table 1 Effects of experimental treatments on parasite densities (means ± se)

	Host and parasite schedules		Route of infection		Stage injected		Rsq
	Matched	*Mismatched*	*IP*	*IV*	*Rings*	*Trophozoites*	
Day 1 pi	1.02 ± 0.01	0.84 ± 0.01	0.72 ± 0.10	1.12 ± 0.09	1.13 ± 0.10	0.71 ± 0.09	0.433
Day 2 pi	3.76 ± 0.67	2.59 ± 0.35	2.07 ± 0.30	4.29 ± 0.62	4.31 ± 0.61	2.05 ± 0.30	0.458
Cumulative	2.06 ± 0.12	1.38 ± 0.12	1.51 ± 0.14	1.93 ± 0.13	1.99 ± 0.14	1.45 ± 0.11	0.591

Rsq values for minimal models are included. Note, there was no significant difference between matched or mismatched parasites on day 1 pi. Day 1 and 2 pi are $\times 10^6$ /mL and cumulative densities are $\times 10^9$ /mL.

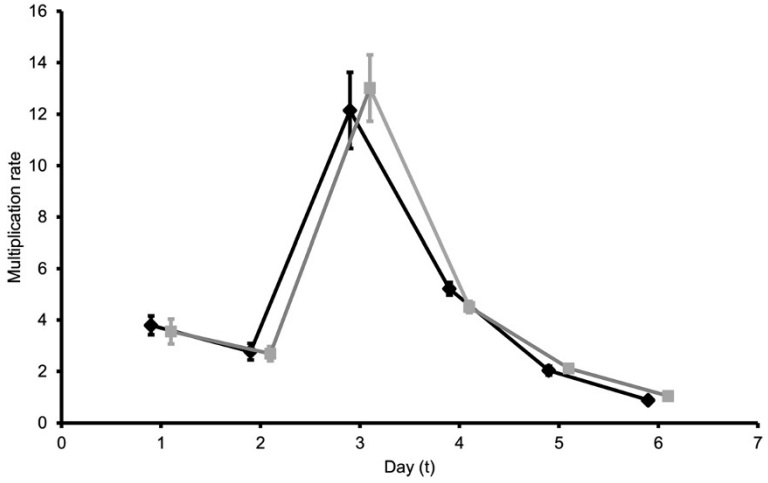

Figure 5 Multiplication rate (number of progeny produced per parasite). The means (±se) for matched (black lines) and mismatched (grey lines) infections are plotted for each cycle of replication (data plotted on the x-axis are offset for clarity), calculated as the number of parasites observed on day $t + 1$ divided by the number on the previous day (t). For example, data plotted on day 1 represent the multiplier between day 1 to day 2.

What host circadian processes could act on parasites in the initial stage of infection only? Given that the cost of mismatch is independent of the route of infection and that it may manifest between day 1–2 pi (when the IP-injected parasites have appeared in the blood) processes operating in the bloodstream are likely responsible. An intriguing possibility is that mismatch between the recipient host and the rhythm of the donor RBC, rather than the parasites themselves, generates an early cost. Recent work has demonstrated that RBCs have their own circadian rhythms, driven by the redox state of the cell [28,29]. If the mismatch between the donor RBC's state and the recipient host's rhythm leads to these cells being preferentially filtered by the spleen or targeted by housekeeping immune responses, then this would generate an early cost for mismatched parasites. However, many components of the mammalian immune system in the blood and spleen exhibit circadian periodicity [17,30-37], so if these are involved in clearing unwanted RBC we would not expect to see costs in both mismatched treatment (since these processes are unlikely to be at their peak activity in both the host's morning and night). However, whether parasitised RBC maintain a normal redox rhythm and hosts can discriminate the RBC redox state of either the infected and/or uninfected RBC present in the inocula, regardless of whether they are injected in the morning or evening, is unknown. If such mechanisms exist, the progeny of parasites that survived the first day in the bloodstream would infect a host RBC on the correct schedule, and thus would not subsequently suffer from the same cost.

Another possibility is that dead parasites/RBC in the inocula – but not the ongoing live infection – provide a transient extra stimulation for innate effectors with circadian schedules. Both this and the RBC redox state explanation are unconvincing because their effects are likely to be apparent on day 1 pi. A more plausible scenario is that parasites must exceed a density threshold to activate early innate responses (e g, a density that is achieved after day 1 in this experiment) and that these responses can be overwhelmed at high parasite densities [38]. This would make the cost of mismatch greatest, and perhaps only apparent, at intermediate densities. More work is required to determine whether costs of mismatch were not apparent on day 1 pi due to lack of statistical power. Statistically detecting a small effect requires a large sample size and a multivariate power analysis reveals that with 20 infections per group, as for this experiment, the chance of detecting a significant effect on day 1 pi (given the observed means and variances) is 73%. Therefore, repeating the experiment with larger sample sizes, reducing the variation in density estimates across infections (e.g., by assaying multiple samples per infection each day), and including other infective doses will enable more thorough investigation of the timing of the costs of mismatch.

That the cost of schedule mismatch is not influenced by either the route of infection (IP or IV) or parasite stage (ring or trophozoite) is unexpected. Macrophages line the peritoneal cavity and have an autonomous 24-hour clock that regulates phagocytosis and the rhythmic secretion of TNF and IL-6 in response to infection, with peak activity late in the day [17,35,37]. Parasites administered via IP in the evening were therefore expected to experience a harsher environment than parasites inoculated IP in the morning. Furthermore, late-stage parasites are thought to be more susceptible to stress than rings, as suggested for fever (e.g., heat shock disproportionately kills parasites in the latter half of the cell cycle [39,40]).

Therefore, trophozoite-stage parasites were expected to be more vulnerable to time-of-day effects compared to infections initiated with rings. If such stressors included active macrophages then inoculation of trophozoites in the evening via IP would result in the poorest performing infections. This is not the case because trophozoites are not disproportionately disadvantaged by time, nor route, of infection.

Conclusions

It is beneficial for parasites to be in synchrony with their host's rhythm, regardless of the route of infection or the parasite stage inoculated. The data presented here suggest mismatch impacts on the ability of parasites to establish infections, but not on their ability to multiply, and that the reduction in 'starting number' has a magnifying effect on density as infections progress. While the coordination between parasites and host rhythms is apparent, whether this is actively achieved by the parasite or passively established by host rhythms remains unknown. Because hosts infected by mismatched parasites experience less severe anaemia, hosts would benefit by causing parasites to become mismatched. Hosts do not appear to do this, suggesting that hosts are not in control of parasite schedules, or that host rhythms are unavoidably responsible for parasite schedules. How parasites benefit from synchronisation with the host, and why this is particularly important at the start of infections, also remains unknown. The answers to these questions may be revealed by identifying whether parasite stages differ in their vulnerability to circadian innate effectors, if parasites have resource requirements that are only met at certain times of day, how these processes are affected by parasite density, and whether the costs of mismatch vary across different durations of time shift. Given that arrested cell-cycle development (quiescence) is implicated in tolerance to drugs [41-45], understanding what governs these schedules as well as the costs and benefits of adjusting them is important.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AOD and SR conceived and designed the project; AOD carried out the experiment; all authors analysed the data and prepared the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank P Schneider and G K P Barra for assistance, L Pollitt and D Kennedy for discussion, the Wissenschaftskolleg zu Berlin for a Fellowship (SR), and the Royal Society, the Wellcome Trust (082234), the Centre for Immunity, Infection and Evolution (095831), and an NSERC Discovery Grant (NM) for funding.

Author details

[1]Institutes of Evolution, Immunology and Infection Research, University of Edinburgh, Edinburgh, UK. [2]Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Canada. [3]Centre for Immunity, Infection and Evolution, University of Edinburgh, Edinburgh, UK.

Received: 23 August 2013 Accepted: 23 October 2013
Published: 26 October 2013

References

1. Dodd AN, Salathia N, Hall A, Kevei E, Toth R, Nagy F, Hibberd JM, Millar AJ, Webb AAR: **Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage.** *Science* 2005, **309:**630–633.
2. Ouyang Y, Andersson CR, Kondo T, Golden SS, Johnson CH: **Resonating circadian clocks enhance fitness in cyanobacteria.** *Proc Natl Acad Sci USA* 1998, **95:**8660–8664.
3. Saunders DS: **Circadian control of larval growth rate in *Sarcophaga argyrostoma*.** *Proc Natl Acad Sci USA* 1972, **69:**2738–2740.
4. Green RM, Tingay S, Wang ZY, Tobin EM: **Circadian rhythms confer a higher level of fitness to Arabidopsis plants.** *Plant Physiol* 2002, **129:**576–584.
5. Sawa M, Nusinow DA, Kay SA, Imaizumi T: **FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis.** *Science* 2007, **318:**261–265.
6. Xu KY, DiAngelo JR, Hughes ME, Hogenesch JB, Sehgal A: **The circadian clock interacts with metabolic physiology to influence reproductive fitness.** *Cell Metab* 2011, **13:**639–654.
7. Yerushalmi S, Yakir E, Green RM: **Circadian clocks and adaptation in Arabidopsis.** *Mol Ecol* 2011, **20:**1155–1165.
8. Killick-Kendrick R, Peters W: *Rodent malaria.* London: Academic Press; 1978.
9. Mideo N, Reece SE, Smith AL, Metcalf CJ: **The Cinderella syndrome: why do malaria-infected cells burst at midnight?** *Trends Parasitol* 2013, **29:**10–16.
10. O'Donnell AJ, Schneider P, McWatters HG, Reece SE: **Fitness costs of disrupting circadian rhythms in malaria parasites.** *Proc Biol Sci* 2011, **278:**2429–2436.
11. Bell AS, De Roode JC, Sim D, Read AF: **Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success.** *Evolution* 2006, **60:**1358–1371.
12. Mackinnon MJ, Read AF: **Immunity promotes virulence evolution in a malaria model.** *PLoS Biol* 2004, **2:**1286–1292.
13. Reece SE, Ali E, Schneider P, Babiker HA: **Stress, drugs and the evolution of reproductive restraint in malaria parasites.** *Proc Biol Sci* 2010, **277:**3123–3129.
14. Schneider P, Bell AS, Sim DG, O'Donnell AJ, Blanford S, Paaijmans KP, Read AF, Reece SE: **Virulence, drug sensitivity and transmission success in the rodent malaria, *Plasmodium chabaudi*.** *Proc Biol Sci* 2012, **279:**4677–4685.
15. Pollitt LC, Mideo N, Drew DR, Schneider P, Colegrave N, Reece SE: **Competition and the evolution of reproductive restraint in malaria parasites.** *Am Nat* 2011, **177:**358–367.
16. Gautret P, Deharo E, Tahar R, Chabaud AG, Landau I: **The adjustment of the schizogonic cycle of *Plasmodium chabaudi chabaudi* in the blood to the circadian rhythm of the host.** *Parasite* 1995, **2:**69–74.
17. Keller M, Mazuch J, Abraham U, Eom GD, Herzog ED, Volk HD, Kramer A, Maier B: **A circadian clock in macrophages controls inflammatory immune responses.** *Proc Natl Acad Sci U S A* 2009, **106:**21407–21412.
18. Mackinnon MJ, Read AF: **Selection for high and low virulence in the malaria parasite *Plasmodium chabaudi*.** *Proc Biol Sci* 1999, **266:**741–748.
19. Schneider P, Chan BHK, Reece SE, Read AF: **Does the drug sensitivity of malaria parasites depend on their virulence?** *Malar J* 2008, **7:**257.
20. Vitaterna MH, King DP, Chang AM, Kornhauser JM, Lowrey PL, McDonald JD, Dove WF, Pinto LH, Turek FW, Takahashi JS: **Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior.** *Science* 1994, **264:**719–725.
21. Mackinnon MJ, Read AF: **Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*.** *Evolution* 1999, **53:**689–703.
22. Boyd GH: **Induced variations in the asexual cycle of *Plasmodium cathemerium*.** *Am J Hyg* 1929, **9:**181–187.
23. Boyd GH: **Experimental modification of the reproductive activity of *Plasmodium cathemerium*.** *J Exp Zool* 1929, **54:**111–126.
24. Taliaferro WH, Taliaferro LG: **Alteration in the time of sporulation of *Plasmodium brasilianum* in monkeys by reversal of light and dark.** *Am J Epidemiol* 1934, **20:**50–59.
25. Wargo AR, de Roode JC, Huijben S, Drew DR, Read AF: **Transmission stage investment of malaria parasites in response to in-host competition.** *Proc Biol Sci* 2007, **274:**2629–2638.
26. **The R foundation for statistical computing.** http://www.R-project.org.
27. Pollitt LC, Reece SE, Mideo N, Nussey DH, Colegrave N: **The problem of auto-correlation in parasitology.** *PLoS Pathog* 2012, **8:**e1002590.

28. Bass J, Takahashi JS: **Circadian rhythms redox redux.** *Nature* 2011, **469:**476–478.
29. O'Neill JS, Reddy AB: **Circadian clocks in human red blood cells.** *Nature* 2011, **469:**498–U470.
30. Arjona A, Sarkar DK: **Circadian oscillations of clock genes, cytolytic factors, and cytokines in rat NK cells.** *J Immunol* 2005, **174:**7618–7624.
31. Bollinger T, Bollinger A, Naujoks J, Lange T, Solbach W: **The influence of regulatory T cells and diurnal hormone rhythms on T helper cell activity.** *Immunology* 2010, **131:**488–500.
32. Bollinger T, Bollinger A, Skrum L, Dimitrov S, Lange T, Solbach W: **Sleep-dependent activity of T cells and regulatory T cells.** *Clin Exp Immunol* 2009, **155:**231–238.
33. Bollinger T, Leutz A, Leliavski A, Skrum L, Kovac J, Bonacina L, Benedict C, Lange T, Westermann J, Oster H, Solbach W: **Circadian clocks in mouse and human CD4+ T cells.** *PLoS One* 2011, **6:**e29801.
34. Haus E, Smolensky MH: **Biologic rhythms in the immune system.** *Chronobiol Int* 1999, **16:**581–622.
35. Hayashi M, Shimba S, Tezuka M: **Characterization of the molecular clock in mouse peritoneal macrophages.** *Biol Pharm Bull* 2007, **30:**621–626.
36. Scheff JD, Calvano SE, Lowry SF, Androulakis IP: **Modeling the influence of circadian rhythms on the acute inflammatory response.** *J Theor Biol* 2010, **264:**1068–1076.
37. Silver AC, Arjona A, Walker WE, Fikrig E: **The circadian clock controls toll-like receptor 9-mediated innate and adaptive immunity.** *Immunity* 2012, **36:**251–261.
38. Metcalf CJE, Graham AL, Huijben S, Barclay VC, Long GH, Grenfell BT, Read AF, Bjornstad ON: **Partitioning regulatory mechanisms of within-host malaria dynamics using the effective propagation number.** *Science* 2011, **333:**984–988.
39. Kwiatkowski D: **Febrile temperatures can synchronize the growth of *Plasmodium falciparum* in vitro.** *J Exp Med* 1989, **169:**357–361.
40. Kwiatkowski D, Greenwood BM: **Why is malaria fever periodic? A hypothesis.** *Parasitol Today* 1989, **5:**264–266.
41. Codd A, Teuscher F, Kyle DE, Cheng Q, Gatton ML: **Artemisinin-induced parasite dormancy: a plausible mechanism for treatment failure.** *Malar J* 2011, **10:**56.
42. Witkowski B, Lelievre J, Barragan MJ, Laurent V, Su XZ, Berry A, Benoit-Vical F: **Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism.** *Antimicrob Agents Chemother* 2010, **54:**1872–1877.
43. Cambie G, Caillard V, Beaute-Lafitte A, Ginsburg H, Chabaud A, Landau I: **Chronotherapy of malaria: identification of drug-sensitive stage of parasite and timing of drug delivery for improved therapy.** *Ann Parasitol Hum Comp* 1991, **66:**14–21.
44. Francois G, Chimanuka B, Timperman G, Holenz J, Plaizier-Vercammen J, Ake Assi L, Bringmann G: **Differential sensitivity of erythrocytic stages of the rodent malaria parasite *Plasmodium chabaudi chabaudi* to dioncophylline B, a highly active naphthylisoquinoline alkaloid.** *Parasitol Res* 1999, **85:**935–941.
45. Klonis N, Xie SC, McCaw JM, Crespo-Ortiz MP, Zaloumis SG, Simpson JA, Tilley L: **Altered temporal response of malaria parasites determines differential sensitivity to artemisinin.** *Proc Natl Acad Sci U S A* 2013, **110:**5157–5162.

doi:10.1186/1475-2875-12-372
Cite this article as: O'Donnell *et al.*: Disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated. *Malaria Journal* 2013 **12**:372.

MALARIA JOURNAL

CORRECTION Open Access

Correction: disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated

Aidan J O'Donnell[1*], Nicole Mideo[2] and Sarah E Reece[1,3]

Correction

Some of the data in the article [1] were inadvertently mislabelled. Specifically, for infections initiated with trophozoite stage parasites, the schedule "matched" treatment group was incorrectly analysed as "mismatched" and vice-versa. The data have been re-analysed and the effects of perturbing the schedules of parasites relative to the host circadian rhythm are more complex than presented in the original paper. However, the differences between initiating infections with ring stages versus trophozoite stages, and via intraperitoneal injection or intravenous injection remain unchanged. The affected sections of the paper (data analysis method, results, discussion) have been re-written and new figures drawn. The authors apologize for any inconvenience or confusion that this may have caused.

Data analysis

R version 2.6.1 (The R foundation for statistical computing; http://www.R-project.org; Vienna, Austria) was used for all analyses. General Linear Models were used to test how the perturbations of the route of infection, parasite stage, and co-ordination of parasite and host rhythms affected (i) the ability of parasites to establish infections (days 1 and 2 pi) and (ii) their overall performance to the peak of infections (cumulative density between days 1–7). Data for day 2 post-infection were $\log_{10}$ transformed to conform to the assumptions of normality. General linear mixed effects models were used to examine whether replication rate was affected by mismatch of host and parasite rhythms. This required fitting mouse identity as random effect to control for the non-independence of multiple data points from each infection [2]. Maximal models contained all main effects and interactions, and models were minimised using stepwise deletion until only significant terms remained.

Results

The route of infection, parasite stage, and mismatch between host and parasite schedules all had significant effects on parasite densities (Figure 1, replaces Figure three). The influence of these factors varied across infections and explained between 42-59% (R^2) of variation in parasite numbers.

On day 1 (Figure 1a), infections via IV had significantly higher densities than via IP ($F_{(1,\ 36)}$ =12.90; P <0.001) and infections initiated with rings performed significantly better than infections initiated with trophozoites ($F_{(1,\ 36)}$ =13.40; P <0.001; R^2 = 0.42). However, the densities of matched and mismatched parasite densities did not differ significantly ($F_{(1,\ 36)}$ =0.22; P =0.640). On Day 2 (Figure 1b), there were significant interactions between route of infection and parasite stage ($F_{(1,\ 34)}$ =5.04; P =0.031) and between parasite schedule and parasite stage ($F_{(1,\ 34)}$ =5.84; P =0.021; R^2 = 0.52). Infections initiated with rings always had higher densities than infections initiated with trophozoites, and this difference was greatest when the route of infection was IP. Mismatch had a substantial negative effect on infections initiated with rings but not trophozoites (R^2 = 0.52). These effects became more pronounced over the pre-peak phase of the infection (Figure 1c; R^2 = 0.59): mismatch was costly (1.4 fold reduction) for infections initiated with rings but beneficial (1.6 fold increase) to those initiated with trophozoites ($F_{(1,\ 35)}$ =5.84; P =0.021), and higher parasite densities were always observed in infections via IV compared to IP ($F_{(1,\ 35)}$ =9.82; P =0.003).

Hosts lost RBCs throughout the pre-peak phase of the infection and the patterns mirrored parasite performance (Figure 1d; R^2 = 0.52). Hosts infected via IV lost significantly more RBC (i e, had greater anaemia) than via IP ($F_{(1,\ 35)}$ =22.32; P <0.001). Again, there was a significant interaction between schedule and stage ($F_{(1,\ 35)}$ =6.35; P =0.016) in which hosts infected with matched trophozoites lost the least RBC.

* Correspondence: Aidan.Odonnell@ed.ac.uk
[1]Institutes of Evolution, Immunology and Infection Research, University of Edinburgh, Edinburgh, UK
Full list of author information is available at the end of the article

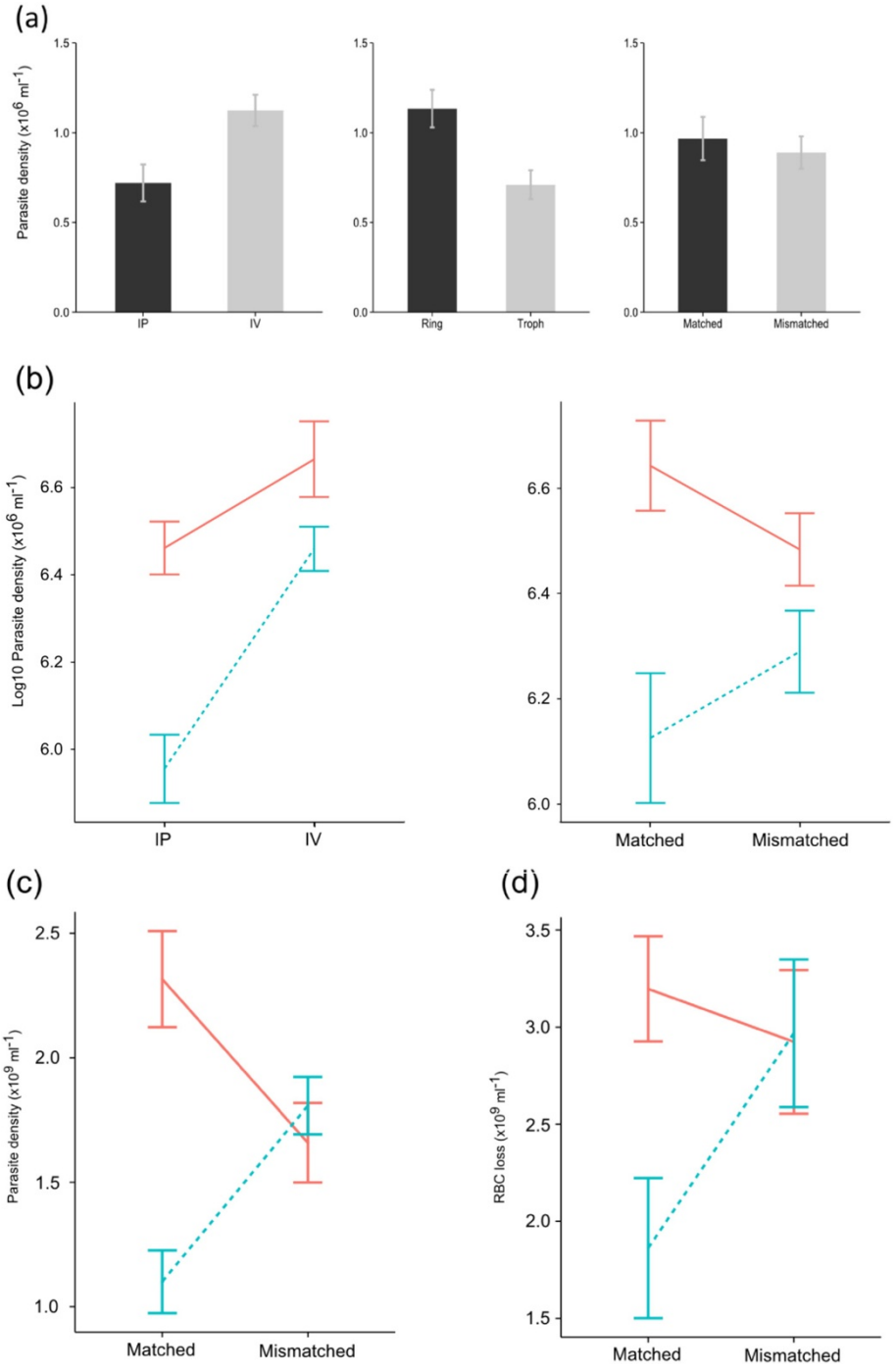

Figure 1 From Day 2 the impact of mismatch varies based on which parasite stage initiated the infection. (a) Parasite densities of infections on Day 1 post-infection. Bars show mean (±se) densities of parasites with n =39 infections. The left plot compares the route of infection either by IP (intraperitoneal injection, black bars) or IV (intravenous injection, grey bars). The middle plot compares the parasite stage used to initiate the infections, with rings (black bars) and trophozoites (grey bars). The right plot compares parasites on the same (matched, black bars) or perturbed (mismatched, grey bars) schedule as the host. Parasite stage (rings, solid lines; trophozoites, dotted lines) and whether parasites were matched or mismatched to the host schedule had significant effects on Day 2 post infection **(b)** and across the pre-peak phase **(c).** Mean (±se) densities are plotted (note for **(b)** the analysis required the data to be transformed). The mean (±se) amount of RBC lost hosts depended on the stage and schedule of parasites they were infected with **(d).** n =40 infections for **(b) – (d).**

The number of progeny produced by each parasite (multiplication rate) varied during infections ($\chi^2_5 = 263.32$; P <0.001) but did not differ significantly between matched and mismatched parasites, for all replication cycles examined (Schedule: $\chi^2_1 = 0.302$; P =0.582) (Figure 2, replaces Figure five). This result, taken together with the significant difference in densities appearing by day 2 pi suggests that circadian processes operating in the initial phase of infection affect parasite number in a stage-specific manner (benefit trophozoites and harm rings) and this initial difference is propagated throughout infections to result in significant effects of mismatch with the host rhythm.

It is easy to show algebraically that any small difference in parasite densities, such as the difference observed between matched and mismatched parasites by day 1 post infection, will increase at a rate proportional to the multiplication rate, even when each parasite produces the same number of progeny per cell cycle. If the initial densities of matched and mismatched parasites are p and $p + \varepsilon$, respectively, and the multiplication rate of all parasites is r, then after t days (rounds of replication) the density of matched and mismatched parasites will be $r^t p$ and $r^t(p + \varepsilon)$ and the difference in densities between matched and mismatched infections will have increased by a factor of r^t (i e, from ε to $r^t\varepsilon$). Even if multiplication rates change over time (i e, r changes over time, as is the case; Figure 2), as long as it is greater than 1, the difference between matched and mismatched parasite densities will increase as infections progress.

Discussion

This experiment involved the simultaneous perturbation of coordination between host and parasite schedules, the stage of parasite inoculated, and the route of infection. The data show that mismatch to host rhythms is costly for *P. chabaudi* parasites regardless of the route of infection, but reveal that this phenomena depends on the developmental stage inoculated. The experiment also revealed that, as expected, ring stage parasites are generally more successful in establishing infections than trophozoite stages (which is presumably why, conventionally, ring stages are used to initiate experimental infections) and both stages benefit from being injected straight into the blood stream rather than having to negotiate their way from the peritoneal cavity to the blood (by an as yet unknown mechanism). Finally, the interaction between co-ordination of parasite and host rhythms and parasite stage may have consequences for virulence because mice in infected with matched trophozoite stages suffer less anaemia than mice in the other treatment groups.

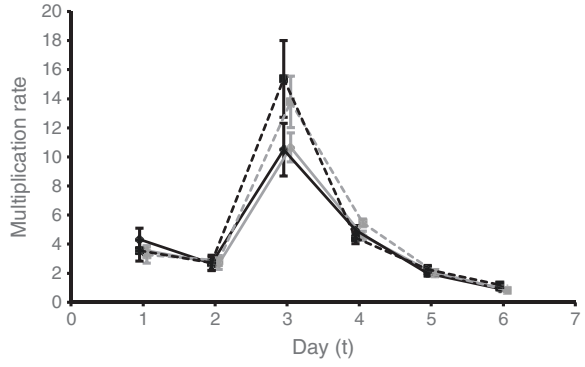

Figure 2 Multiplication rate (number of progeny produced per parasite). The means (±se) for matched (black lines) and mismatched (grey lines) infections initiated by rings (solid lines) and trophozoites (dotted lines) are plotted for each cycle of replication (the x-axis is offset for clarity), calculated as the number of parasites observed on day t +1 divided by the number on the previous day (t). For example, data plotted on day 1 represent the multiplier between day 1 to day 2.

This experiment, coupled with previous work [3], confirm that a phase-shift of between nine to 12 hours is detrimental for ring stage parasites and unexpectedly reveal that phase-shift is beneficial for trophozoite stage parasites. Moreover, further analyses reject the hypothesis that the costs of mismatch are due to processes that reduce the multiplication rate of parasites throughout infections, but instead, suggest that processes operating when parasites are establishing a blood stage infection are responsible. The lack of impact of time-of-day effects throughout infections cannot be explained by parasite schedules quickly adjusting to become synchronised with the host circadian rhythm. Staging parasites in blood smears verified that 3 days after inoculation parasites were maintaining their original developmental schedule (data not shown), and previous work suggests that if adjustment occurs, it takes at least 7 days [3-7].

Why might ring stage parasites suffer from schedule mismatch whereas trophozoite stages benefit? One explanation is that it is simply costly for parasites to enter the host in the evening (when mismatched ring stages and matched trophozoites were inoculated, Figure 3). Given that these costs are independent of the route of infection and that costs manifest between day 1–2 pi (when the IP-injected parasites have appeared in the blood) processes operating in the bloodstream are likely responsible. Many components of mammalian blood, including RBC [2,8], and immune factors in the blood and spleen exhibit circadian periodicity and often appear to be upregulated in the dark phase of the day [9-17]. However, whether such responses would only impact on parasites in the first 1 or 2 days post infection is unknown. There may be immune responses that are short acting, upregulated in the dark phase, directed against parasites, and that can be overwhelmed above a threshold parasite density [18]. Or, an immune response that is only effective at low densities

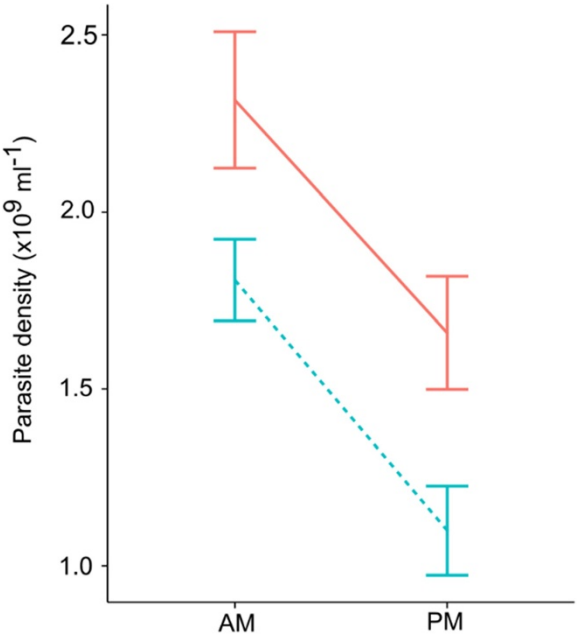

Figure 3 Performance of parasites entering the host in the evening and morning. Re-plot of data in Figure 1c showing mean (±se) densities achieved during the pre-peak phase of infections by different stages (rings, solid lines; trophozoites, dotted lines).

may only be active during the first few bouts of parasite replication (schizogony). Alternatively, if some immune response(s) are upregulated in the dark phase and directed towards anomalous RBC, then RBC from donor mice may be recognised and cleared by this process. In this case, once parasites have undergone schizogony they reside in the host's own RBC and escape this process on all subsequent days.

That the effect of schedule mismatch is not influenced by the route of infection (IP or IV) is unexpected. Macrophages line the peritoneal cavity and have an autonomous 24-hour clock that regulates phagocytosis and the rhythmic secretion of TNF and IL-6 in response to infection, with peak activity late in the day [9,15,17]. Parasites – at any stage - administered via IP in the evening were, therefore, expected to experience a harsher environment than parasites inoculated IP in the morning. Furthermore, late-stage parasites are thought to be more susceptible to stress than rings, as suggested for fever (e g, heat shock disproportionately kills parasites in the latter half of the cell cycle [19,20]). If such stressors included active macrophages then trophozoites would be more vulnerable than rings when inoculated in the evening via IP. This is not the case because whilst trophozoites perform better when inoculated in the morning, this was not restricted to the IP group (i e, the 3-way interaction between schedule, stage, and route was not significant).

Conclusions

It is beneficial for infections initiated with ring stage parasites to be in synchrony with their host's rhythm and for trophozoites to be out of sync, regardless of the route of infection. The data presented here suggest mismatch impacts on the ability of ring stage parasites to establish infections, but not on their ability to multiply, and that the reduction in 'starting number' has a magnifying effect on density as infections progress. How different parasite stages are affected by synchronisation with the host, and why this is particularly important at the start of infections, also remains unknown. The answers to these questions may be revealed by directly testing whether parasite stages differ in their vulnerability to circadian innate effectors, if parasites have resource requirements that are only met at certain times of day, and how these processes are affected by parasite density. Unravelling the mechanisms that explain the differential effects of mismatch is necessary to determine whether the synchronicity and schedules of *P. chabaudi* cell cycles is under the control of parasites or hosts. Given that arrested cell-cycle development (quiescence) is implicated in tolerance to drugs [21-25], understanding what governs these schedules as well as the costs and benefits of adjusting them is important.

Acknowledgements

The authors thank P Schneider and G K P Barra for assistance, L Pollitt and D Kennedy for discussion, the Wissenschaftskolleg zu Berlin for a Fellowship (SR), and the Royal Society, the Wellcome Trust (082234), the Centre for Immunity, Infection and Evolution (095831), the HFSP, and an NSERC Discovery Grant (NM) for funding.

Author details

[1]Institutes of Evolution, Immunology and Infection Research, University of Edinburgh, Edinburgh, UK. [2]Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Canada. [3]Centre for Immunity, Infection and Evolution, University of Edinburgh, Edinburgh, UK.

Received: 28 October 2014 Accepted: 5 November 2014
Published: 17 December 2014

References

1. O'Donnell AJ, Mideo N, Reece SE: **Disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated.** *Malar J* 2013, **12:**372.
2. O'Neill JS, Reddy AB: **Circadian clocks in human red blood cells.** *Nature* 2011, **469:**498–U470.
3. O'Donnell AJ, Schneider P, McWatters HG, Reece SE: **Fitness costs of disrupting circadian rhythms in malaria parasites.** *Proc Biol Sci* 2011, **278:**2429–2436.
4. Gautret P, Deharo E, Tahar R, Chabaud AG, Landau I: **The adjustment of the schizogonic cycle of *Plasmodium chabaudi chabaudi* in the blood to the circadian rhythm of the host.** *Parasite* 1995, **2:**69–74.
5. Boyd GH: **Induced variations in the asexual cycle of *Plasmodium cathemerium*.** *Am J Hyg* 1929, **9:**181–187.
6. Boyd GH: **Experimental modification of the reproductive activity of *Plasmodium cathemerium*.** *J Exp Zool* 1929, **54:**111–126.
7. Taliaferro WH, Taliaferro LG: **Alteration in the time of sporulation of *Plasmodium brasilianum* in monkeys by reversal of light and dark.** *Am J Epidemiol* 1934, **20:**50–59.
8. Bass J, Takahashi JS: **Circadian rhythms redox redux.** *Nature* 2011, **469:**476–478.

9. Keller M, Mazuch J, Abraham U, Eom GD, Herzog ED, Volk HD, Kramer A, Maier B: **A circadian clock in macrophages controls inflammatory immune responses.** *Proc Natl Acad Sci U S A* 2009, **106:**21407–21412.
10. Arjona A, Sarkar DK: **Circadian oscillations of clock genes, cytolytic factors, and cytokines in rat NK cells.** *J Immunol* 2005, **174:**7618–7624.
11. Bollinger T, Bollinger A, Naujoks J, Lange T, Solbach W: **The influence of regulatory T cells and diurnal hormone rhythms on T helper cell activity.** *Immunology* 2010, **131:**488–500.
12. Bollinger T, Bollinger A, Skrum L, Dimitrov S, Lange T, Solbach W: **Sleep-dependent activity of T cells and regulatory T cells.** *Clin Exp Immunol* 2009, **155:**231–238.
13. Bollinger T, Leutz A, Leliavski A, Skrum L, Kovac J, Bonacina L, Benedict C, Lange T, Westermann J, Oster H, Solbach W: **Circadian clocks in mouse and human CD4+ T cells.** *PLoS One* 2011, **6:**e29801.
14. Haus E, Smolensky MH: **Biologic rhythms in the immune system.** *Chronobiol Int* 1999, **16:**581–622.
15. Hayashi M, Shimba S, Tezuka M: **Characterization of the molecular clock in mouse peritoneal macrophages.** *Biol Pharm Bull* 2007, **30:**621–626.
16. Scheff JD, Calvano SE, Lowry SF, Androulakis IP: **Modeling the influence of circadian rhythms on the acute inflammatory response.** *J Theor Biol* 2010, **264:**1068–1076.
17. Silver AC, Arjona A, Walker WE, Fikrig E: **The circadian clock controls toll-like receptor 9-mediated innate and adaptive immunity.** *Immunity* 2012, **36:**251–261.
18. Metcalf CJE, Graham AL, Huijben S, Barclay VC, Long GH, Grenfell BT, Read AF, Bjornstad ON: **Partitioning regulatory mechanisms of within-host malaria dynamics using the effective propagation number.** *Science* 2011, **333:**984–988.
19. Kwiatkowski D: **Febrile temperatures can synchronize the growth of *Plasmodium falciparum* in vitro.** *J Exp Med* 1989, **169:**357–361.
20. Kwiatkowski D, Greenwood BM: **Why is malaria fever periodic? A hypothesis.** *Parasitol Today* 1989, **5:**264–266.
21. Codd A, Teuscher F, Kyle DE, Cheng Q, Gatton ML: **Artemisinin-induced parasite dormancy: a plausible mechanism for treatment failure.** *Malar J* 2011, **10:**56.
22. Witkowski B, Lelievre J, Barragan MJ, Laurent V, Su XZ, Berry A, Benoit-Vical F: **Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism.** *Antimicrob Agents Chemother* 2010, **54:**1872–1877.
23. Cambie G, Caillard V, Beaute-Lafitte A, Ginsburg H, Chabaud A, Landau I: **Chronotherapy of malaria: identification of drug-sensitive stage of parasite and timing of drug delivery for improved therapy.** *Ann Parasitol Hum Comp* 1991, **66:**14–21.
24. Francois G, Chimanuka B, Timperman G, Holenz J, Plaizier-Vercammen J, Ake Assi L, Bringmann G: **Differential sensitivity of erythrocytic stages of the rodent malaria parasite *Plasmodium chabaudi chabaudi* to dioncophylline B, a highly active naphthylisoquinoline alkaloid.** *Parasitol Res* 1999, **85:**935–941.
25. Klonis N, Xie SC, McCaw JM, Crespo-Ortiz MP, Zaloumis SG, Simpson JA, Tilley L: **Altered temporal response of malaria parasites determines differential sensitivity to artemisinin.** *Proc Natl Acad Sci U S A* 2013, **110:**5157–5162.

doi:10.1186/1475-2875-13-503
Cite this article as: O'Donnell *et al.*: Correction: disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated. *Malaria Journal* 2014 **13**:503.

Paper #3 (2019): Time-of-day of blood-feeding: effects on mosquito life history and malaria transmission

Parasites & Vectors

RESEARCH Open Access

Time-of-day of blood-feeding: effects on mosquito life history and malaria transmission

Aidan J. O'Donnell[1*], Samuel S. C. Rund[2] and Sarah E. Reece[1]

Abstract

Background: Biological rhythms allow organisms to compartmentalise and coordinate behaviours, physiologies, and cellular processes with the predictable daily rhythms of their environment. There is increasing recognition that the biological rhythms of mosquitoes that vector parasites are important for global health. For example, whether perturbations in blood foraging rhythms as a consequence of vector control measures can undermine disease control. To address this, we explore the impacts of altered timing of blood-feeding on mosquito life history traits and malaria transmission.

Methods: We present three experiments in which *Anopheles stephensi* mosquitoes were fed in the morning or evening on blood that had different qualities, including: (i) chemical-induced or (ii) *Plasmodium chabaudi* infection-induced anaemia; (iii) *Plasmodium berghei* infection but no anaemia; or (iv) stemming from hosts at different times of day. We then compared whether time-of-day variation in blood meal characteristics influences mosquito fitness proxies relating to survival and reproduction, and malaria transmission proxies.

Results: Mosquito lifespan is not influenced by the time-of-day they received a blood meal, but several reproductive metrics are affected, depending on other blood characteristics. Overall, our data suggest that receiving a blood meal in the morning makes mosquitoes more likely to lay eggs, lay slightly sooner and have a larger clutch size. In keeping with previous work, *P. berghei* infection reduces mosquito lifespan and the likelihood of laying eggs, but time-of-day of blood-feeding does not impact upon these metrics nor on transmission of this parasite.

Conclusion: The time-of-day of blood-feeding does not appear to have major consequences for mosquito fitness or transmission of asynchronous malaria species. If our results from a laboratory colony of mosquitoes living in benign conditions hold for wild mosquitoes, it suggests that mosquitoes have sufficient flexibility in their physiology to cope with changes in biting time induced by evading insecticide-treated bed nets. Future work should consider the impact of multiple feeding cycles and the abiotic stresses imposed by the need to forage for blood during times of day when hosts are not protected by bed nets.

Keywords: Biological rhythm, Circadian rhythm, Fitness, Reproduction, Survival, Fecundity, *Plasmodium berghei*, *Plasmodium chabaudi*, *Anopheles stephensi*

*Correspondence: aidan.odonnell@ed.ac.uk
[1] Institute of Evolutionary Biology, and Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh, UK
Full list of author information is available at the end of the article

Background

Daily rhythms are a ubiquitous feature of life [1]. For example, circadian clocks are thought to enable organisms to coordinate with environmental periodicity in factors such as light/dark, humidity, UV exposure [2]. Interactions with predators, prey and hosts (in the case of parasites) also follow daily rhythms [3–5]. How daily rhythms, whether they are clock-controlled or direct responses to rhythmic environmental cues, shape, and are shaped by interactions between organisms is poorly understood. We address this by examining the consequences of daily rhythms in the interactions between vectors, their hosts, and their parasites. Specifically, we ask how the time-of-day that mosquitoes blood feed combines with the timing (phase) of rhythms in hosts and with malaria infection to shape vector fitness and disease transmission. Given reports that some mosquito populations have altered the time-of-day they bite (likely in response to the use of insecticide-treated bed nets) [6–13], exploring the consequences of perturbed blood foraging rhythms for mosquito fitness and malaria transmission is urgently required.

Mosquitoes exhibit periodicity in many fitness determining activities, including sugar feeding, the formation of mating swarms, insecticide resistance and blood-feeding [14, 15]. In keeping with this, ~20% of the *Anopheles gambiae* genome is expressed in patterns following daily rhythms [16]. Thus, the circadian clock enables mosquitoes to coordinate the timing of the physiological, cellular and molecular processes that underpin behaviours, with rhythms in the abiotic environment and/or other internal processes [2, 17]. For example, *Anopheline* mosquitoes are primarily night-biters [15, 18, 19] and processes associated with being active and foraging at night, including glycolysis, energy sensing and nutrient mobilization are upregulated in concert [16, 20]. Many genes, however, are not clock-regulated but still follow daily rhythms (including some *An. gambiae* odorant-binding proteins) and are driven by a direct response to light or dark [21]. Indeed in both *An. gambiae* and *Aedes aegypti*, more rhythmic genes are detected under light:dark conditions than dark:dark conditions [21, 22].

A key benefit of clock-control is that organisms can anticipate dawn/dusk and prepare in advance by up- or downregulating physiological processes. For example, processes required to cope with a blood meal are upregulated in the mosquito's active phase (night time for *Anopheles* sp.) [14, 16]. This includes catalase and other factors used to detoxify reactive oxygen species (ROS) generated as a product of blood (heme) digestion, and members of the V-ATPase complex which drive water excretion to minimise the 3-fold increase in volume that a blood meal brings [23, 24]. Exposure to ROS increases mortality and reduces clutch size of mosquitoes [23, 25, 26]. Further, as a consequence of the detoxification of blood meal induced ROS, there is a proliferation of mosquito gut microbiota [27] which have complex interactions with parasite infection [28] that may vary in line with time-of-day a blood meal is taken. In addition to rhythms in processes associated with foraging, the activities and locations of immune effectors cycle throughout the day. For example, immune defences are upregulated during the day in diurnal insects, such as *Drosophila* [29, 30]. Whether immune defences peak at night in nocturnal mosquitoes is unknown but some immune genes implicated in interactions with malaria parasites are expressed with circadian rhythms [16]. How circadian rhythms in insect immune defences relate to protection from infection or the severity of disease is unclear. For instance, *Drosophila* challenged with *Pseudomonas aeruginosa* at night are more likely to survive the infection than those challenged in the day. However, perturbation of clock genes to generate arrhythmic mutant flies can result in both decreased survival or enhanced survival depending on the specific genes modified [31]. Further, there are complex consequences of challenging *An. stephensi* with *E. coli* or the malaria parasite *P. chabaudi* at different times of day [32, 33].

Given the potential for circadian rhythms to influence the ability of mosquitoes to cope with a blood meal and with parasites, the time-of-day that mosquitoes forage has implications for both mosquito fitness and disease transmission. These consequences are likely to be complex [14]. If feeding in the daytime means that mosquitoes are less able to cope with the osmotic and oxidative costs of blood, their fecundity and survival should suffer. Indeed, mosquitoes in poor condition as a consequence of feeding in the day may have compromised immune defence and this might explain recent observations that day-fed *An. stephensi* harbour higher densities of *P. chabaudi* than night-fed mosquitoes (although parasite rhythms also mediate this effect) [33]. Alternatively, ROS is a key player in insect immune responses and so, if day-fed mosquitoes do not manage their ROS efficiently, they may suffer collateral damage but also benefit from enhanced parasite defence. Furthermore, it is also necessary to recognise that mosquitoes feed on hosts that have their own circadian rhythms [14]. This includes rhythms in red blood cell composition and density, hematocrit, amino acid composition and immune effectors [34–39]. Thus, rhythms in the composition of mammalian blood could exacerbate (or reduce) the effects of a daytime blood meal on mosquito survival and fecundity.

Clearly, predicting the net effects of how host rhythms and vector rhythms interact to shape malaria transmission is challenging but important. Such interactions

could shape the probability and intensity of infection in mosquitoes as well as mosquito population dynamics. Here, three experiments are described that probe the consequences, under a variety of scenarios, of time-of-day-specific blood-feeding for proxies estimating the fitness of mosquitoes and malaria parasites. The aims are to determine: (i) if the timing of a blood meal affects mosquito survival and fecundity; (ii) whether the effects of time-of-day are exacerbated by other characteristics of host blood or malaria infection; and (iii) the consequences of blood-feeding at different times of day for malaria transmission.

Methods

All experiments examine metrics of mosquito fecundity and lifespan in response to perturbing the time-of-day (morning) or (evening) that mosquitoes receive a blood meal, but differ in the following respects. The first experiment ("*blood quality and host time*", Fig. 1a) includes the effects of both host time-of-day and feeding on blood from anaemic *versus* control mice. To further probe a role for blood quality, the second experiment ("*blood quality*", Fig. 1b) uses a different approach to examine the effects of feeding on anaemic blood but does not consider host time-of-day. The third experiment ("*infection*," Fig. 1c) focuses on *Plasmodium berghei* infection of mosquitoes.

Mice

For all experiments, hosts were 10–12-week-old MF1 male mice housed at 21 °C with *ad libitum* food and drinking water supplemented with 0.05% para-aminobenzoic acid (to supplement parasite growth). Mice were housed in groups of five in either 12:12 light:dark (LD; lights on at 07:00 GMT, lights off at 19:00 GMT) or inverted dark:light photocycle (DL; lights on at 19:00 GMT, lights off at 07:00 GMT) depending on the experiment. Mice were entrained to their respective light schedules for at least 21 days prior to mosquito blood feeds. Prior to donating a blood meal, each mouse was anaesthetized (17% Dormitor, 13% Vetelar, 70% PBS administered at 4 µl/g) and then exposed to a single cage of mosquitoes.

Mosquitoes

All *Anopheles stephensi* mosquitoes were maintained under standard insectary conditions of 27 ± 1 °C, 70% relative humidity and a 12:12 light:dark photocycle, with lights on at 07:00 GMT (ZT0) and lights off at 19:00 GMT (ZT12) (ZT0, Zeitgeber Time 0, is defined as time of lights on). Larvae were reared at a density of ~ 250 larvae per 1.5 l of distilled water. Between 12 and 14 days after hatching, pupae were transferred to emergence cages in incubators (27 ± 1 °C, 60 ± 5% relative humidity) with one-hour light ramping to simulate a dawn (starting at 07:00 GMT; ZT0) and dusk (19:00 GMT; ZT12). Mosquitoes were supplied with *ad libitum* access to 10% fructose solution supplemented with 0.05% paraminobenzoic acid. In the second experiment only, mosquitoes were treated with antibiotics (0.05% gentamicin) administered via their fructose solution 4–5 days before blood meals. For all experiments, female mosquitoes were randomly selected from 3–4 emergence cages, transferred to 2 l holding cages and starved of fructose solution for 24 h before their blood meals. Cages contained 15–85 mosquitoes (depending on the sampling regime of each experiment). Regardless of mosquito number, all mosquitoes were able to blood feed until satiated. For all feeds, each cage of females was exposed to an anaesthetized mouse for 30 min in a light setting that matched the mosquito time-of-day (i.e. morning-fed mosquitoes were fed during lights on and evening feeds were performed under dim red light). Unfed females were removed from the cages (< 5 per cage in all cases). After feeding, mosquitoes were housed in incubators at temperatures of either 20.5 or 26.0 °C (± 0.5 °C), depending on the experiment.

Experimental designs

Experiment 1: blood quality and host time

Mosquito cages were randomly assigned to receive a blood meal in their morning 09:00 GMT (ZT2) or evening 21:00 GMT (ZT14). These feed times are analogous to the mosquito resting period (morning) or active period (evening) as evident from wild caught and laboratory-based studies (Fig. 2, [40–42]). Within each feeding time, cages were allocated to a further four groups, based on host treatment (anaemic or control mice) and host time-of-day [morning mice (ZT2) or evening mice (ZT14)]. The availability of mice experiencing their morning or evening to feed to mosquitoes in their morning or evening was achieved by housing mice in room with LD and DL lighting schedules. This resulted in an experiment with a $2 \times 2 \times 2$ design: eight groups varying by feed time (morning/evening), host blood treatment (anaemic/control), and host time (morning/evening) (Fig. 1a). Note, this is the only experiment that perturbs host time-of-day.

Anaemia was induced in half of the mice by intraperitoneal injection of 125 mg/kg of phenylhydrazine 3 days before feeding to mosquitoes. The control mice received a sham injection of 100 µl PBS. On the day of feeding, red blood cell (RBC) counts ($\times 10^9$ ml^{-1}) for control mice (7.48 ± 0.13 SE) were almost 2-fold higher than for phenylhydrazine treated hosts (3.80 ± 0.12 SE; $t = 21.27$, $df = 45.67$, $P < 0.001$). Each cage contained 50 mosquitoes and each of the 8 treatment groups contained 6 cages. On day 2 post-blood meal (PBM), mosquitoes were allocated

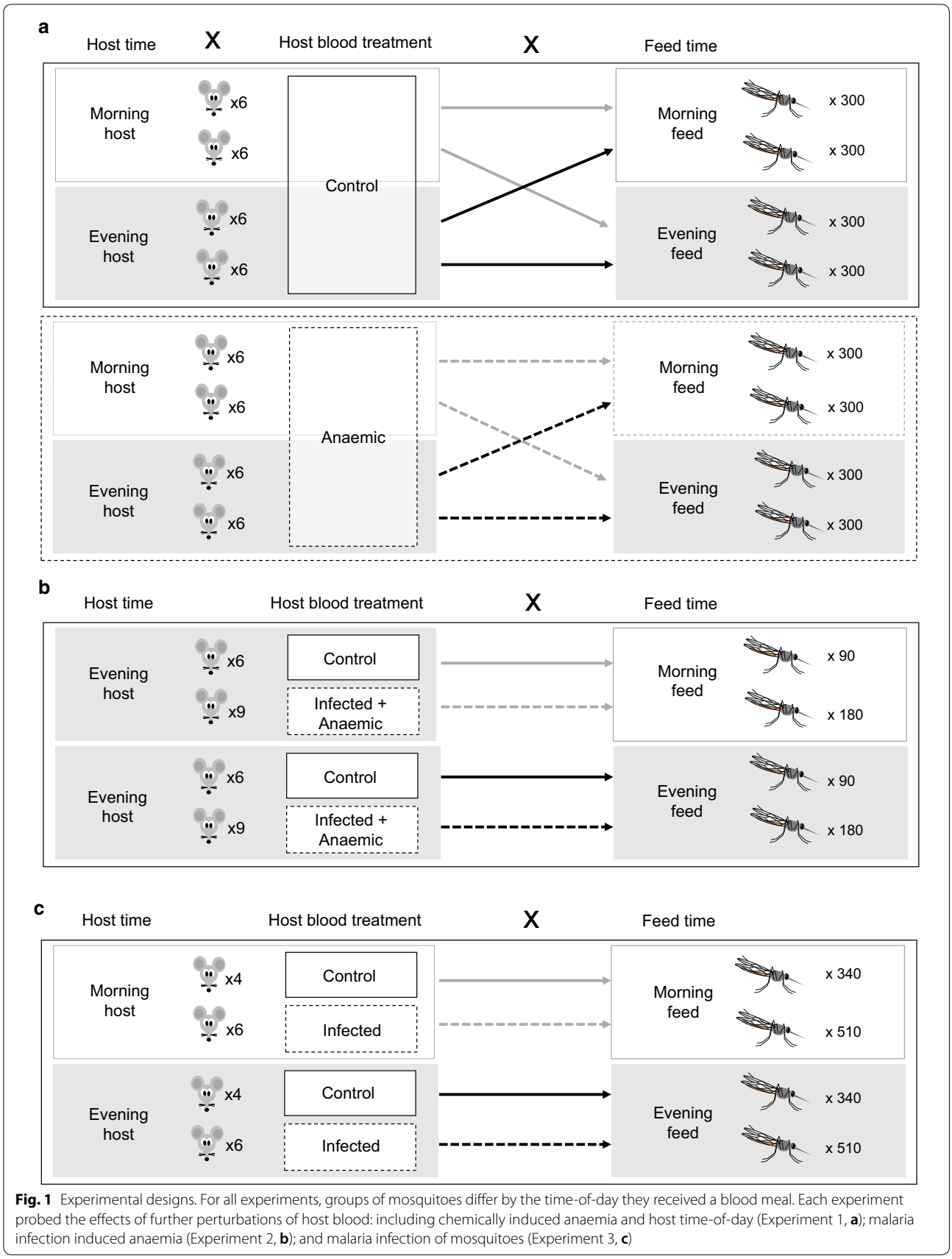

Fig. 1 Experimental designs. For all experiments, groups of mosquitoes differ by the time-of-day they received a blood meal. Each experiment probed the effects of further perturbations of host blood: including chemically induced anaemia and host time-of-day (Experiment 1, **a**); malaria infection induced anaemia (Experiment 2, **b**); and malaria infection of mosquitoes (Experiment 3, **c**)

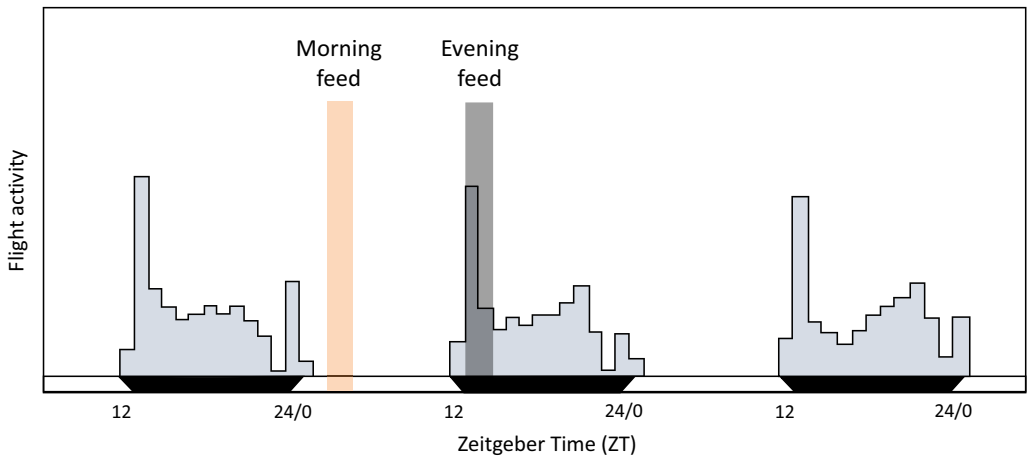

Fig. 2 Blood feed timing. Daily flight activity of lab reared *An. stephensi* mated females (modified from [42]) showing that mosquito flight activity peaks after dusk (ZT12) with a second smaller peak before dawn (ZT0). Wild-caught mosquitoes also show this pattern, with slight variations to the size of the dusk peak depending on monsoon season [40, 41]. Shading represents timing of morning (orange) and evening (grey) blood meals in experiments 1–3. This placed the evening feeds during the mosquito's active period and the morning feeds during the mosquito's rest period

to individual housing (50 ml falcon tubes with *ad libitum* access to 10% glucose solution *via* a 1.5 ml microcentrifuge tube feeder). Fecundity and mortality were tracked for 20 mosquitoes from each cage (960 total). Each female was given a 30 mm diameter Petri dish lined with filter paper and 3 mm depth of distilled water as an oviposition habitat. Mosquitoes were checked daily and if eggs were present, the egg dish was replaced. Egg bowls were photographed at the time of removal (for clutch size counts) incubated for 6 days and then photographed again so that all hatched larvae (alive and dead) could be counted (to estimate hatch rate). For all females (that did or did not lay) egg bowls were removed on day 9 PBM.

Additionally, the volume and density of blood meals were estimated for 10 randomly chosen mosquitoes from each cage (480 total) 2 hours after their blood meal. The right wing of each mosquito was photographed, and the abdomen removed and homogenised in 500 µl drabkins solution for ~30 min [43]. Samples were split into two 200 µl sub-samples and optical density (OD) read by a spectrophotometer at 540 nm (each mosquito was read in duplicate, and an average taken). To generate control series for each cage, 8 µl of blood was removed from each mouse used to feed mosquitoes at the time of feeding and used to generate 4 µl, 1 µl, 0.8 µl and 0.4 µl standards. Host RBC density readings (cells per µl) were also obtained at the time of feeding to calculate the RBC density of the blood meal. Wing length was obtained from the photographs, converted to mm and used to control for any potential differences in blood meal volume and density due to variation in body size (using the software package ImageJ [44]).

Experiment 2: blood quality

Here, instead of phenylhydrazine treatment, blood quality was perturbed by using malaria infection to generate anaemia. Mosquito cages were randomly assigned to morning 09:00 GMT (ZT2) or evening 21:00 GMT (ZT14) feed times. At each feeding time, half the cages were exposed to anaemic or (uninfected) control mice. This resulted in an experiment with a 2×2 design: four groups varying by the timing of their blood meal (morning/evening) and blood treatment (anaemic/control) (Fig. 1b). Note, host time-of-day was standardised by housing mice in two rooms with inverted light schedules (DL and LD), enabling both the morning- and evening-fed mosquitoes to feed on hosts experiencing their evening (host ZT14). Six cages were fed, at each time point, on control mice and nine cages, at each time point, on anaemic mice. Each cage exposed to control mice contained 15 mosquitoes and each cage exposed to anaemic mice contained 20 mosquitoes. Mortality was tracked as for Experiment 1 (but for 10 individuals per cage; 300 total) and egg dishes were provided until day 14 PBM.

All feeds occurred on mice at day 11 post-infection (PI) after infection with 1×10^6 *P. chabaudi* CR parasitized RBCs or sham infection (controls; 100 µl PBS). *Plasmodium chabaudi* has a synchronous asexual cycle so donor mice were used from each room (DL and LD) to ensure that all hosts were infected with rings (i.e. parasite and host rhythms were phase matched; [45]). By day 11 PI, significant anaemia had occurred (mean RBC density $\times 10^9$ ml^{-1}: Control = 7.88 (±0.16 SE), anaemic = 4.44 (±0.10 SE); $t = 18.77$, $df = 19.28$, $P < 0.001$) and hosts were mounting strong immune responses,

so the parasite was not able to establish an infection in mosquitoes (parasite mating is very vulnerable to sub-optimal conditions in the blood meal [46]). Thus, mosquitoes received poor quality blood as a result of a more ecologically realistic perturbation than PHZ, without the confounding effects of becoming infected themselves. This was verified by examining 10 randomly selected mosquitoes from each cage exposed to anaemic mice on day 14 PBM. Specifically, the midgut of each mosquito was dissected, stained for two minutes in 0.5% mercurochrome, washed in PBS and total oocysts per midgut were counted *via* microscopy. No oocysts were detected.

Experiment 3: infection

Mosquito cages were randomly assigned to four groups. Two groups received their blood meal in the morning 10:00 GMT (morning, ZT3) and the others were fed in the evening at 20:00 GMT (evening, ZT13). At each feed time, half of the cages were exposed to *Plasmodium berghei* infected mice or naïve (uninfected) control mice. This resulted in an experiment with a 2×2 design: four groups varying by the timing of their blood meal (morning/evening) and blood treatment (infected/uninfected) (Fig. 1c). Note that time-of-day for parasites/hosts and mosquitoes is synonymous; morning-fed mosquitoes received blood from hosts also experiencing their morning, and *vice versa* for evening-fed mosquitoes. At each time point, six cages were fed on infected mice and four fed cages on uninfected mice.

All feeds occurred on mice at day 6 PI after inoculation with 1×10^5 *P. berghei* parasitized RBCs or sham infection (controls; 100 µl PBS). Infections (and sham injections) were staggered by 10 h to ensure that morning- and evening-fed mosquitoes were exposed to infections of the same age (144 h). *Plasmodium berghei* was chosen because its asexual cycle is asynchronous, ensuring that morning- and evening-fed mosquitoes did not receive significantly different stage distributions of asexual parasites (feed time:parasite stage; $\chi^2_4 = 1.28$, $P = 0.29$; Fig. 3a) or gametocyte densities/ages (mean gametocyte density $\times 10^7$ ml^{-1}: morning = 3.93 (± 0.14 SE), evening = 3.80 (± 0.1 SE); $t = 0.77$, $df = 10$, $P = 0.46$; Fig. 3b). On day 6 PI, *P. berghei* had not significantly reduced the RBC density of hosts (mean RBC density $\times 10^9$ ml^{-1}: Control = 8.17 (± 0.08 SE), Infected = 7.85 (± 0.18 SE); $t = 1.64$, $df = 14.71$, $P = 0.12$).

At the time of feeding, each cage contained 85 mosquitoes. After the blood meal, 15 mosquitoes from cages fed on infected mice were removed (180 total) and used to monitor oocyst prevalence and density as for Experiment 2. To track mosquito fecundity and mortality, a subset of 15 randomly selected females were removed from each cage (300 total) 2 days PBM and housed individually in 200 ml cups with *ad libitum* access to 10% fructose solution. On day 3 PBM, each female was given a 30 mm diameter Petri dish lined with filter paper and 3 mm depth of distilled water as an oviposition habitat. Mosquitoes were checked daily until death, if eggs were present the egg dish was replaced (up until day 21 PBM).

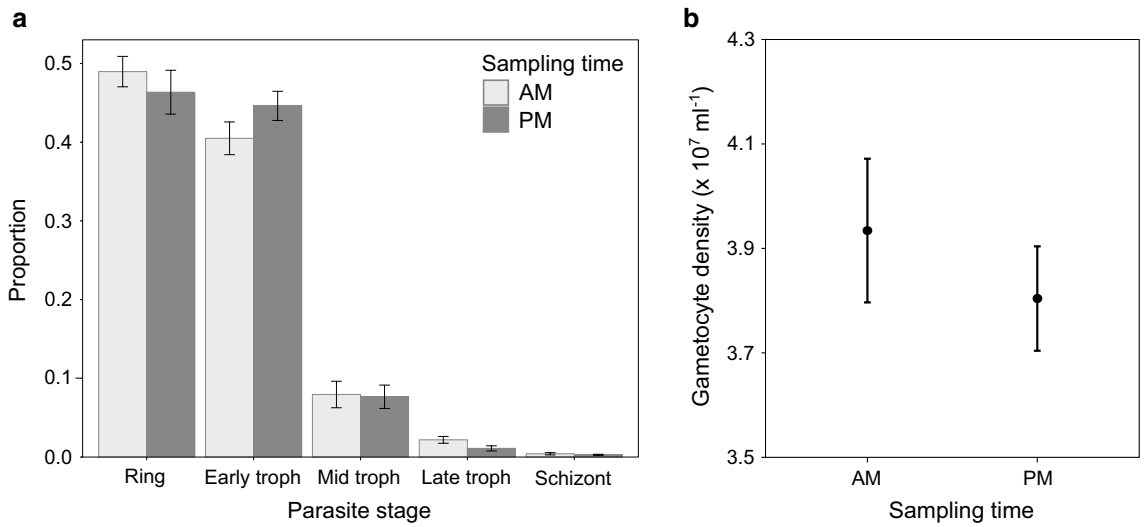

Fig. 3 Composition of *P. berghei* parasites in mice used to infect mosquitoes in experiment 3. Shown are the means ± SE parasite stage proportions (**a**) and gametocyte density (**b**) from infected hosts used for blood meals. Donors were sampled at the time of blood meals either in the morning (AM; 10:00 GMT; ZT3) or evening (PM; 20:00 GMT; ZT13) ($n = 6$)

Data analysis

R version 2.6.1 [47] was used for all analyses. Model simplification was carried out by stepwise deletion of the least significant term and only minimal models are reported. Measurements made from mice at the time of feeding (red blood cell counts and parasite stage composition and densities), and time-of-day differences in infection load for mosquitoes were analysed with Student's t-test. Mosquito fecundity metrics, proportion of females that laid and hatch rate, were analysed using generalised linear mixed-effects models with binomial error structures. Clutch size and blood meal measures were analysed using linear mixed effect models. In both types of linear models, identity of the mosquito cage was included as a random effect. All models met model assumptions: independence of data points, normality of residuals and homogeneity of variances (confirmed through assessing the model plots, the Shapiro–Wilk test and Bartlett's test). Cox proportional hazard models with mosquito identity nested within cage as random effects (frailty model) were used to estimate the effects of feed time and host blood manipulations on the time taken to lay and lifespan (*coxme* package in R [48]). All Cox models model met the proportional hazards assumptions based on Schoenfeld's residuals (evaluated using the 'cox.zph' function R; $P > 0.1$ for all variables). Clutch size of mosquitoes that laid and its interactions with experimental treatments was also controlled for because the data indicated considerable heterogeneity in clutch size, and trade-offs between survival and reproduction have been reported [49, 50] and may depend on resource availability, which may vary as a consequence of perturbations of blood quality. For this reason, mosquitoes that did not lay eggs were excluded from time to lay, clutch size and lifespan analyses. For all analyses, main effects and two-way interactions were investigated.

Results

We carried out three experiments to determine how the timing of receiving a blood meal affects aspects of mosquito survival and fecundity, and whether qualities of host blood or malaria infection modulate the effects of the time-of-day that mosquitoes feed.

Experiment 1: blood quality and host time

This experiment (Fig. 1a) recognises that hosts have circadian rhythms in blood composition and was designed to address if host time-of-day and blood quality (chemical induced anaemia) interact with mosquito feeding time-of-day to shape the following parameters (see Table 1 for a summary).

Blood meal: volume and density

There was no significant effect of feed time ($\chi^2_7 = 1.02$, $P = 0.31$), host time ($\chi^2_5 = 0.01$, $P = 0.91$), or their interaction ($\chi^2_9 = 0.40$, $P = 0.53$) on the volume of the blood meal. The effect of host blood quality was not significantly influenced by interactions with feed time ($\chi^2_8 = 0.75$, $P = 0.39$) or host time ($\chi^2_6 = 2.29$, $P = 0.13$). However, mosquitoes that fed on anaemic hosts took up a greater volume of blood than those that fed on control hosts (mean ± SE blood meal volume (µl) per mm wing length: control = 0.26 ± 0.01), anaemic = 0.33 ± 0.01; $\chi^2_4 = 17.90$, $P < 0.0001$; Fig. 4a). There was also a borderline significant interaction between host time and host blood quality on the RBC density of the blood meal ($\chi^2_6 = 4.30$, $P = 0.038$; Fig. 4b). Specifically, mosquitoes that fed on control hosts consumed more RBCs than those that fed on anaemic hosts, especially when fed on hosts that experienced their morning (mean ± SE × 10^6; control hosts: morning = 2.11 ± 0.94, evening = 1.77 ± 0.75; anaemic hosts: morning = 1.24 ± 0.54, evening = 1.29 ± 0.60). There was no significant effect of feed time ($\chi^2_7 = 0.66$, $P = 0.42$), nor its interactions with host blood quality ($\chi^2_9 = 0.65$, $P = 0.42$) or host time ($\chi^2_8 = 3.25$, $P = 0.07$) on the RBC of the blood meal.

Reproduction: proportion laid

Neither host blood quality ($\chi^2_5 = 1.60$, $P = 0.21$), host time ($\chi^2_4 = 2.44$, $P = 0.12$), or their interaction ($\chi^2_8 = 0.02$, $P = 0.89$) significantly affected the probability each mosquito laid. However, feed time did matter, with mosquitoes that fed in the morning more likely to lay than those that fed in the evening (mean ± SE proportion of females that laid: morning = 0.82 ± 0.02, evening = 0.65 ± 0.02; $\chi^2_3 = 27.56$, $P < 0.0001$; Fig. 5a). However, feed time did not significantly interact with either host blood quality ($\chi^2_6 = 0.41$, $P = 0.52$) or host time ($\chi^2_7 = 0.05$, $P = 0.83$).

Reproduction: time to lay

For mosquitoes that laid, neither host blood quality ($z = 0.73$, $P = 0.47$) or host time ($z = 0.95$, $P = 0.34$) influenced the time it took mosquitoes to lay eggs. Feed time did have an effect with mosquitoes that fed in the morning laying sooner than those that fed in the evening (mean ± SE days taken to lay since egg bowls were provided: morning = 1.15 ± 0.03, evening = 1.44 ± 0.04; evening:morning HR = 0.64 ± 0.08, $z = -5.35$, $P < 0.001$; Fig. 5a).

Reproduction: clutch size

Clutch size was shaped by a borderline interaction between feed time and host blood quality ($\chi^2_6 = 4.13$, $P = 0.042$; Fig. 5b). Mosquitoes fed on control mice had

Table 1 Summary of statistical results for analyses in Experiment 1, Experiment 2 and Experiment 3

Fitness metric	Statistical results for each term in model
Experiment 1	
Blood meal volume	*Sig: Blood quality*
	Non-Sig: Feed time:host time; Feed time:blood quality; Host time:blood quality; Feed time; Host time
Blood meal density	*Sig: Host time:blood quality*
	Non-Sig: Feed time:host time; Feed time:blood quality; Feed time
Proportion laid	*Sig: Feed time*
	Non-Sig: Host time:blood quality; Feed time:host time; Feed time:blood quality; Host time; Blood quality
Time to lay	*Sig: Feed time*
	Non-Sig: Host time; Blood quality
Clutch size	*Sig: Feed time:blood quality*
	Non-sig: Feed time:host time; Host time:blood quality; Host time
Hatch rate	*Sig: na*
	Non-sig: Feed time:host time; Feed time:blood quality; Host time:blood quality; Feed time; Host time; Blood quality
Lifespan	*Sig: na*
	Non-sig: Host time; Feed time; Blood quality
Experiment 2	
Proportion laid	*Sig: na*
	Non-Sig: Feed time:blood quality; Blood quality; Feed time
Time to lay	*Sig: na*
	Non-Sig: Feed time:blood quality; Blood quality; Feed time
Clutch size	*Sig: Blood quality*
	Non-sig: Feed time:blood quality; Feed time
Lifespan	*Sig: na*
	Non-sig: Feed time; Blood quality
Experiment 3	
Malaria prevalence & intensity	*Sig: Infected/uninfected blood*
	Non-Sig: Feed time
Proportion laid	*Sig: Infection status*
	Non-Sig: Infection status:feed time; Feed time
Time to lay	*Sig: Infection status*
	Non-Sig: Infection status:feed time; Feed time
Clutch size	*Sig: na*
	Non-sig: Infection status:feed time; Feed time; Infection status
Lifespan	*Sig: Infection status*
	Non-sig: Feed time

Notes: Terms that significantly affected the mosquito fitness metric in question are highlighted in italics. Interactions between terms are indicated by ':' and main effects are not included for terms involved in significant interactions

higher clutch sizes than those that fed on anaemic hosts, and this difference was greatest when mosquitoes fed in the morning (mean ± SE clutch size: morning-fed: control hosts = 65.57 ± 1.77, anaemic hosts = 51.06 ± 1.70; evening-fed: control hosts = 61.88 ± 1.97, anaemic hosts = 55 ± 2.09). There was also a non-significant tendency for mosquitoes that fed on hosts experiencing their morning to have higher clutch size (mean ± SE clutch size: morning hosts = 56.36 ± 1.37, evening hosts = 59.99 ± 1.34; $\chi^2_7 = 3.70$, $P = 0.054$). However, this trend for an effect of host time was not modulated by feed time ($\chi^2_8 = 3.06$, $P = 0.08$) or host blood quality ($\chi^2_9 = 0.03$, $P = 0.87$).

Reproduction: hatch rate

Neither host blood quality ($\chi^2_4 = 0.12$, $P = 0.73$) or host time ($\chi^2_6 = 0.02$, $P = 0.89$) or their interaction ($\chi^2_8 = 0.10$, $P = 0.75$) influenced egg hatch rate. Likewise, there was no significant influence of feed time ($\chi^2_3 = 1.31$, $P = 0.25$) and its interactions with host blood quality ($\chi^2_5 = 2.12$, $P = 0.15$) and host time ($\chi^2_7 = 0.40$, $P = 0.53$). Mean hatch rate for all clutches was 0.69 (± 0.01 SE).

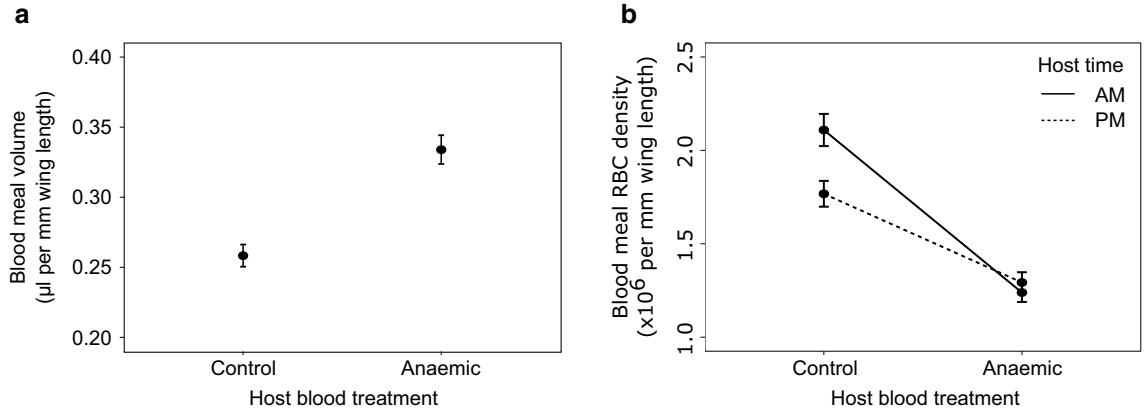

Fig. 4 Blood meal volume (**a**) and blood meal RBC density (**b**) in response to perturbations of host blood quality and host time-of-day (Experiment 1). Mean ± SE, adjusted for body size. AM indicates morning for hosts and parasites, at 09:00 GMT (ZT 2) and PM indicates 21:00 GMT (ZT 14). Anaemia was induced in hosts by phenylhydrazine (125 mg/kg injected intraperitoneally)

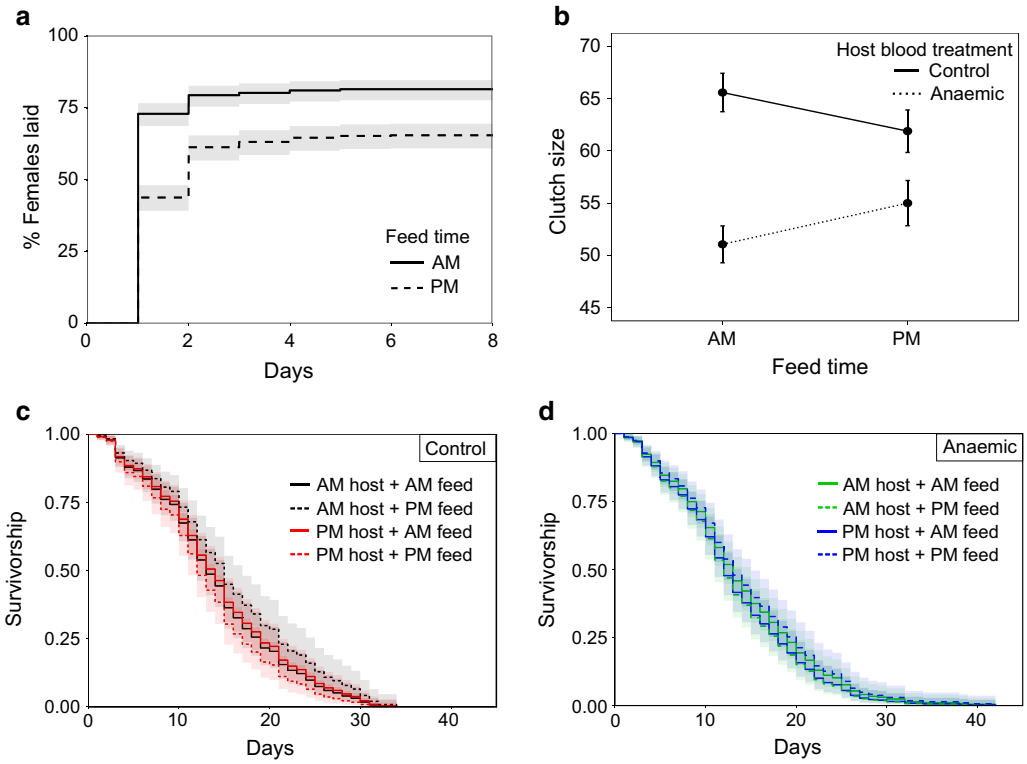

Fig. 5 Mosquito reproduction and survival in response to perturbations of host blood quality and host time-of-day (Experiment 1). Shown are Kaplan–Meier curves for the time taken to lay (**a**), mean ± SE clutch size (**b**), and Kaplan–Meier curves for mosquito survival (**c**, **d**). AM indicates a morning feed at 09:00 GMT (ZT 2) and PM an evening feed at 21:00 GMT (ZT 14). Mosquitoes that did not lay are omitted from **b**, **c** and **d**. Due to the negative effect of clutch size on survival Kaplan–Meier curves for survival (**c** and **d**) were estimated based on median clutch size of 65. Lines represent time to lay event (**a**) and survival estimates with 95% confidence intervals in shading (**c**, **d**). Anaemia was induced in hosts by phenylhydrazine (125 mg/kg injected intraperitoneally)

Lifespan

For mosquitoes that laid, neither host blood quality ($z=0.28$, $P=0.78$), host time ($z=0.75$, $P=0.45$) or feed time ($z=-0.10$, $P=0.92$) significantly influenced mortality rate (Fig. 5c–d). Clutch size was negatively associated with survival hazard (clutch HR = 0.996, $z=-2.54$, $P=0.011$), with smaller clutches (< 60 eggs) associated with a greater hazard than larger clutches (> 60 eggs). The median lifespan for all mosquitoes (that laid) was 13 days post-blood meal.

Experiment 2: blood quality

Experiment 1 suggested that blood quality and mosquito time-of-day of feeding shaped some mosquito reproductive measures (tendency to lay and clutch size). Experiment 2 (Fig. 1b) further investigated time-of-day of feeding and blood quality by using *P. chabaudi* malaria infection to generate anaemia (see Table 1 for a summary). Host time-of-day was not investigated further because Experiment 1 revealed that it did not significantly shape mosquito reproduction or lifespan (host time-of-day only remained in an interaction with borderline significance for blood meal density).

Reproduction: proportion laid

Neither host blood quality ($\chi^2_4=0.73$, $P=0.39$), feed time ($\chi^2_3=1.59$, $P=0.21$) nor their interaction ($\chi^2_5=1.07$, $P=0.30$) significantly influenced the proportion of females that laid (Fig. 6a). The mean proportion of females that laid per cage was 0.48 (± 0.04 SE).

Reproduction: time to lay

For mosquitoes that laid, neither host blood treatment ($z=0.52$, $P=0.60$), feed time ($z=0.99$, $P=0.32$) nor their interaction ($z=0.74$, $P=0.46$) significantly affected the time taken to lay (Fig. 6a). The average number of days to lay since eggs bowls were provided was 2.63 (± 0.17 SE).

Reproduction: clutch size

Host blood quality significantly affected clutch size, with mosquitoes fed on control blood laying larger clutches than mosquitoes that received anaemic blood (mean ± SE clutch size: control hosts = 90.36 ± 5.06, anaemic hosts = 66.41 ± 3.06; $\chi^2_4=8.62$, $P=0.003$; Fig. 6b). Feed time did not influence clutch size ($\chi^2_5=1.84$, $P=0.17$) or modulate the effect of blood quality ($\chi^2_6=1.81$, $P=0.18$).

Lifespan

For mosquitoes that laid, neither host blood quality ($z=1.05$, $P=0.29$), feed time ($z=-0.98$, $P=0.33$) or clutch size ($z=-1.81$, $P=0.07$) influenced mortality rates (Fig. 6c). The median lifespan was 39 days post-blood meal.

Experiment 3: infection

Having investigated blood quality and host time-of-day in the previous experiments, we switched focus to consider the effects of malaria infection by feeding mosquitoes on blood with infectious *P. berghei* parasites at different times of day (Fig. 1c and Table 1). In addition to the effects on mosquito reproduction and lifespan, the performance of parasites was also examined.

Parasites

No mosquitoes that fed on control hosts became infected but 94 (± 2%) mosquitoes that fed on infected hosts contained oocysts. Within mosquitoes fed on infected hosts, feed time did not influence infection prevalence ($t_{(10)}=0.598$, $P=0.56$; Fig. 7a) or the intensity of infection

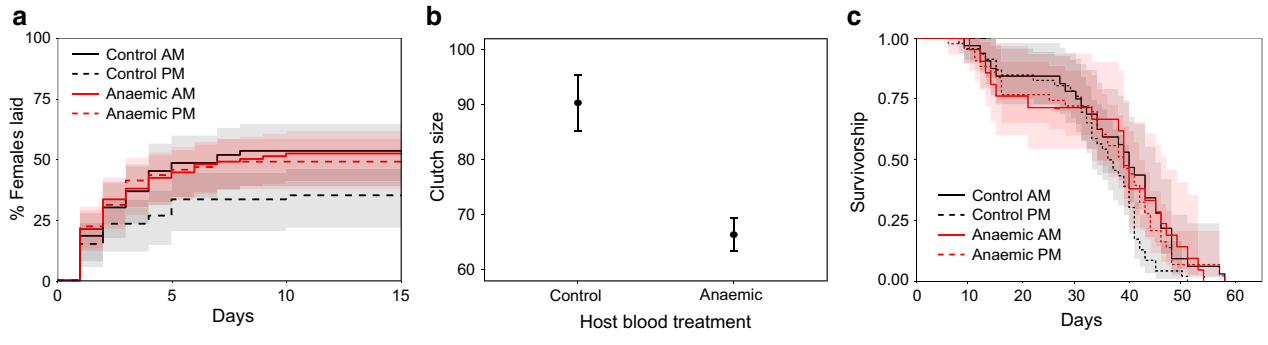

Fig. 6 Mosquito reproduction and survival in response to perturbations of host blood quality (Experiment 2). Shown are Kaplan–Meier curves for the time taken to lay (**a**), mean ± SE clutch size (**b**), and Kaplan–Meier curves for survival (**c**). Mosquitoes that did not lay are omitted from **b** and **c**. Lines represent time to lay event (**a**) and survival estimates with 95% confidence intervals in shading (**c**). AM indicates a blood feed in the morning at 09:00 GMT (ZT 2) and PM a feed in the evening at 21:00 GMT (ZT 14). Anaemia was induced by *P. chabaudi* malaria infection, but the parasites were not infectious to mosquitoes

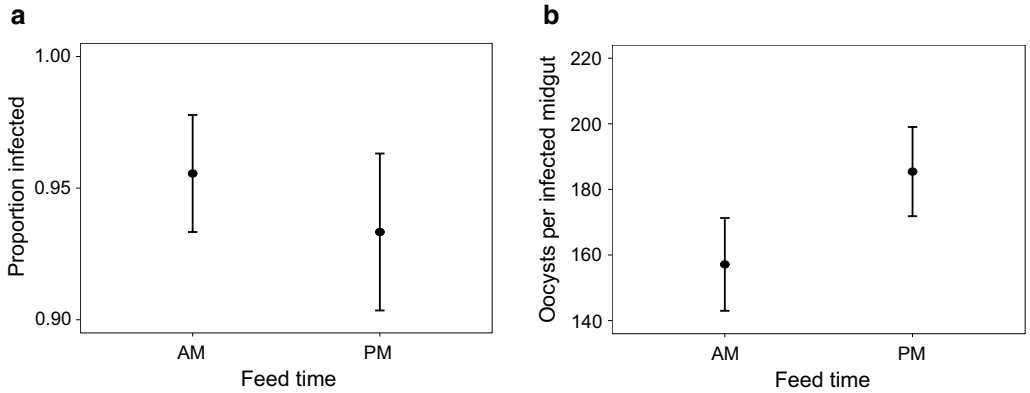

Fig. 7 Mosquito parasite burden and intensity in response to perturbations to blood meal time (Experiment 3). Shown are the mean ± SE proportion of mosquitoes infected with *P. berghei* parasites (**a**) and mean ± SE oocyst burden per infected midgut (**b**). AM indicates a blood feed in the morning at 10:00 GMT (ZT 3) and PM a feed in the evening at 20:00 GMT (ZT 13)

(mean ± SE oocysts = 171.3 ± 9.84; $t_{(178)} = -1.442$, $P = 0.15$; Fig. 7b).

Reproduction: proportion laid

A significantly greater proportion of uninfected than infected mosquitoes laid eggs (mean ± SE proportion laid: uninfected = 0.59 ± 0.04, infected = 0.44 ± 0.04; $\chi^2_3 = 5.44$, $P = 0.0197$; Fig. 8a). The influence of infection was not modulated by feed time ($\chi^2_5 = 0.009$, $P = 0.93$) but there was a trend in which mosquitoes fed in the morning were more likely to lay (mean ± SE morning = 0.56 ± 0.04, evening = 0.44 ± 0.05; $\chi^2_4 = 3.66$, $P = 0.056$; Fig. 8a).

Reproduction: time to lay

For mosquitoes that laid, those that were infected laid two days sooner than uninfected individuals and at any time point, were ~70% more likely to lay (mean ± SE days taken to lay since egg bowls were provided: uninfected = 6.88 ± 0.48, infected = 5.05 ± 0.35; infected:uninfected Hazard Ratio (HR) = 1.71 (± 0.171, $z = 3.12$, $P = 0.002$; Fig. 8a). Neither the interaction between infection status and feed time ($z = 0.28$, $P = 0.78$) nor feed time alone ($z = -1.82$, $P = 0.07$) influenced time to lay.

Reproduction: clutch size

Neither host blood treatment ($\chi^2_4 = 0.59$, $P = 0.44$), feed time ($\chi^2_5 = 0.07$, $P = 0.79$) nor their interaction ($\chi^2_6 = 0.30$, $P = 0.59$) significantly influenced clutch size (Fig. 8b). Females laid an average of 98 (± 2.6 SE) eggs per clutch.

Lifespan

For mosquitoes that laid, infection had a negative effect on lifespan with infected mosquitoes dying sooner than

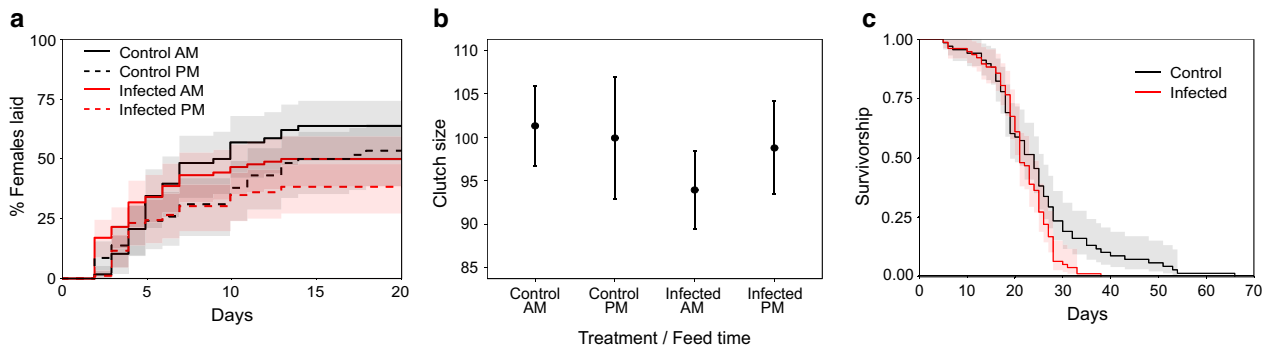

Fig. 8 Mosquito reproduction and survival in response to malaria infection (Experiment 3). Shown are Kaplan–Meier curves for the time taken to lay (**a**), mean ± SE clutch size (**b**), and Kaplan–Meier curves for mosquito survival (**c**). Mosquitoes that did not lay are omitted from **b** and **c**. Lines represent time to lay event (**a**) and survival estimates with 95% confidence intervals in shading (**c**). AM indicates a blood feed in the morning at 10:00 GMT (ZT 3) and PM a feed in the evening at 20:00 GMT (ZT 13). Approximately 95% of infected mosquitoes were parasitized with *P. berghei* whereas control mosquitoes fed on uninfected, healthy hosts

uninfected mosquitoes (median lifespan: infected = 21 days, uninfected = 24 days). Further, infected mosquitoes had a ~50% higher overall hazard of dying (infected:uninfected HR = 1.53 (±0.178 SE), $z = 2.4$, $P = 0.016$; Fig. 8c). Neither feed time ($z = -0.98$, $P = 0.33$) nor clutch size ($z = -0.56$, $P = 0.57$) influenced lifespan.

Discussion

Here, we examine whether the fitness of female *An. stephensi* mosquitoes is affected by the time-of-day they receive a blood meal, either directly or through interactions with perturbations of blood quality and malaria infection. Specifically, we compared mosquitoes fed on control or anaemic hosts (using two different manipulations of anaemia) in which host time-of-day also varied, and whether mosquitoes were uninfected or infected with *P. berghei* malaria. The results of the three experiments are summarised in Table 2 from the perspective of the mosquitoes' time-of-day of feeding. Overall, we found few effects of time-of-day of feeding. First, morning-fed mosquitoes appeared >25% more likely to lay than evening-fed (Figs. 5a, 6a, 8a). Secondly, in response to chemically induced anaemia, morning-fed mosquitoes laid 0.3 of a day sooner (Fig. 5a) and produced ~15 (30%) more eggs than evening-fed mosquitoes (Fig. 5b). However, time-of-day of feeding did not substantially influence longevity of mosquitoes or the prevalence and intensity of *P. berghei* infection (Fig. 7). We also found mixed results for the other variables manipulated in the experiments. Host time-of-day did not influence any of the mosquito fitness metrics we measured. The effects of blood quality were similar across both of the experiments in which it was perturbed (Experiments 1 and 2); only clutch size varied in response, in which mosquitoes fed on anaemic blood laid ~20 (~22%) fewer eggs (Figs. 5b, 6b). Infection status also correlated with fitness metrics; infected mosquitoes had shorter lifespans (21 days post-blood meal for infected *vs* 24 days for uninfected; Fig. 8c), were less likely to lay eggs (44% *vs* 60% laid) and laid sooner (~2 days) than uninfected mosquitoes (Fig. 8a).

We expected that mosquitoes receiving a blood meal at an unexpected time-of-day (i.e. morning) would experience fitness costs in the form of reduced lifespan and/or loss of reproduction. However, we found no effect on lifespan and the effects on reproduction were not consistent with costs; a higher probability of laying eggs and a modest increase in fecundity appear to be fitness benefits from morning feeding (Table 2). Laying sooner may be a fitness cost if it results in poor quality eggs or trade-off against immune defence [51]. However, we found no evidence of a quantity-quality reproductive trade-off because eggs from females in all groups (Experiment 1) hatched at a similar rate. In Experiments 2 and 3 we saw little difference between morning- and evening-fed mosquitoes in the time taken to lay despite the 10-hour 'head-start' of morning-fed mosquitoes. If egg maturation takes a fixed window of time since feeding, this suggests morning-fed mosquitoes are deliberately delaying their oviposition or waiting until the next 'gate' to oviposit if oviposition is clock-controlled. Mark-recapture studies with wild *An. farauti* show that an earlier feed time is associated with irregularities in oviposition cycle, sometimes lengthening or shortening by a day [52–54]. This demonstrates flexibility in the day of oviposition post-blood meal, but whether there is additional flexibility for time-of-day requires further investigation [55, 56]. Given daily mortality risk for mosquitoes (estimated to be around 10% for *An. gambiae* [57]), intuition suggests it would be adaptive to lay as soon as they are able.

A lack of costs of morning feeding could have several non-mutually exclusive explanations. First, costs were expected because the expression of numerous genes involved in processes required to neutralise the ROS produced by blood digestion is rhythmic [16]. However,

Table 2 Summary of statistically significant effects of the time-of-day that mosquitoes blood feed on life history traits

	Experiment 1: "Blood quality & host time"	Experiment 2: "Blood quality"	Experiment 3: "Infection"
Infection prevalence & intensity	na	na	No effect of feed time
Proportion laid	Morning-fed are ~26% more likely to lay than evening-fed	No effect of feed time	Morning-fed are possibly ~27% more likely to lay than evening fed ($P = 0.056$)
Time to lay	Morning-fed are ~1.5 times more likely to lay each day than evening-fed	No effect of feed time	No effect of feed time
Clutch size	Higher if fed on control (non-anaemic) blood in the morning (30% more eggs)	No effect of feed time	No effect of feed time
Hatch rate	No effect of feed time	na	na
Blood meal volume & density	No effect of feed time	na	na
Survival	No effect of feed time	No effect of feed time	No effect of feed time

Abbreviation: na, not available

transcriptional circadian phases do not always reflect protein abundance rhythms [58, 59]. Nine of the 12 V-ATPase subunits of the vesicular type H+ ATPase (V-ATPase), which is associated with maintaining osmotic balance during the increase in volume resulting from a blood meal, are rhythmic at the protein level (peaking at dusk in *An. gambiae* [16]). This has led to the suggestion that water excretion is compromised in mosquitoes feeding in the daytime and so, they should compensate by taking smaller blood meals [14]. However, we found no evidence of feeding time-of-day affecting blood meal volume or density. Secondly, immune responses are suggested to be timed to defend against pathogens acquired during foraging [60]. However, for mosquitoes, there may be an acute need for immune control of the proliferation of gut microbiota that expand upon an influx of blood [61]. ROS favours pathogen defence and a combination of digestion-related and immune-related ROS might erode rhythmicity in ROS levels, or defences may be upregulated as a direct response to feeding, rather than in a time-of-day dependent manner. Thirdly, when only comparing two time points on a symmetrical curve, there is a risk of picking the same intercept as the curve ascends and descends ("shoulder problem"). However, this is unlikely to be the case in our experiments because mosquitoes were in their rest phase in morning feeds and their active phase in evening feeds [42]. Fourthly, if feeding at the wrong time-of-day has only minor negative fitness consequences, manipulating feeding time-of-day over multiple blood-feeding and oviposition cycles might be required to detect costs, or keeping mosquitoes in a more stressful and ecologically realistic manner.

Many nutrients and amino acids in the blood that are essential to mosquito egg development (e.g. isoleucine) [62] exhibit circadian periodicity [34–36] but we found no evidence that host time-of-day matters for mosquitoes feeding on either healthy or anaemic mice. Mice take their largest meal around lights off, and so, by carrying out feeds on mice several hours into their active *versus* rest phases the difference in blood meal composition due to metabolic processes should have been considerable. Perhaps these factors are not limiting at any point in their rhythms, especially for mosquitoes receiving blood from well-fed laboratory mice. Further work could consider investigating the role host time-of-day in more dramatic manipulations of blood composition, for example, during infection and under food-limited conditions.

Our perturbations of anaemia did affect mosquito reproduction; clutch size was reduced in mosquitoes feeding on anaemic blood. Inducing anaemia with phenylhydrazine causes oxidative damage to red blood cells which are then cleared from circulation [63]. ROS damages mosquitoes [23, 25, 26], but by feeding mosquitoes three days after phenylhydrazine administration, the ROS it causes should have been neutralised. Thus, the main difference between blood from control and phenylhydrazine-treated mice is the age structure and density of RBC. Our data suggest that mosquitoes take up a larger volume of blood from phenylhydrazine-treated mice (perhaps facilitated by lower viscosity of anaemic blood [64]), but that this does not fully compensate and equalise blood meal RBC densities to those from feeds on control mice (Fig. 4). There may be additional differences in blood quality between chemical- and infection-induced anaemia. However, given their similar impacts, the ability to garner fewer resources from anaemic blood could explain the reduction in clutch size we observed. This is supported by previously revealed positive correlations between haematin content of blood and clutch size [65, 66]. Additionally, we found that hatch rate is a decreasing function with lay day ($\chi^2_5 = 12.58$, $P < 0.001$) but only in those mosquitoes that fed on anaemic hosts. This result is similar to that reported in infected mosquitoes [67] suggesting that this result may be an effect of blood quality rather than parasite infection.

Our results contrast with recent work showing that mosquitoes' blood-feeding in the daytime are more likely to become infected after feeding on *P. chabaudi* infected mice, although *P. chabaudi* oocyst burdens did not differ between feed times [33]. Compared to *P. berghei*, *P. chabaudi* generally transmits with far lower prevalence and burden, which may facilitate detection of subtle time-of-day effects. An alternative possibility is that mosquito time-of-day effects are driven by an interaction with parasite time-of-day and so, are only observed in infections with synchronously developing parasites such as *P. chabaudi* (in which a specific age of gametocytes is present in blood meals), or in asynchronous species such as *P. relictum* in which parasite abundance in the blood (rather than age) is rhythmic [68]. In contrast to the effects of our other perturbations in the experiments presented here, we found negative effects of infection on lifespan. Costs of malaria infection on mosquito lifespan have been observed in other malaria model systems (reviewed in [69]) as well as an advancement of egg laying [67]. The advanced laying of infected mosquitoes may be a form of terminal investment because organisms with low survival prospects rush to reproduce before dying [70–73]. If our mosquitoes adopted terminal investment, it is necessary to explain why uninfected mosquitoes do not benefit from early reproduction. This could be because advancing reproduction also results in reduced clutch size (but we did not observe this), lower hatch rate (Experiment 1 suggests this does not occur either), trade-offs against anti-parasite immune responses, or reduces the probability or size of future clutches [74].

Alternatively, mosquitoes may restrict essential lipid resources available to parasites by allocating them to eggs as quickly as possible [75] or since mounting an immune response is costly to fecundity, laying early may be a compromise for both fecundity and survival [76].

Conclusions

In summary, we found that taking a blood meal in the morning compared to the evening has no, or minor negative, effects on the fitness of mosquitoes, nor impacts upon on *P. berghei* malaria infection. If our results from a laboratory colony of mosquitoes living in benign conditions hold for wild mosquitoes, it suggests that mosquitoes have sufficient flexibility in their physiology to cope with changes in biting time induced by evading insecticide-treated bed nets. Future work should consider the impact of multiple feeding cycles and the abiotic stresses imposed by the need to forage for blood when hosts are not protected by bed nets.

Acknowledgements
We thank Aidan McHall-Hagan, Iona Brian, Caroline Rodger and G. K. P. Barra for assistance, and Jess Metcalf for help with statistical analysis.

Authors' contributions
AOD and SR conceived and designed the project. AOD carried out the experiments. All authors interpreted the data and prepared the manuscript. All authors read and approved the final manuscript.

Funding
The Royal Society (202769/Z/16/Z; 204511/Z/16/Z), the Human Frontier Science Programme (RGP0046/2013), and the Wellcome (202769/Z/16/A) funded the work.

Availability of data and materials
The datasets supporting the conclusions of this article are available in the Edinburgh DataShare repository, https://doi.org/10.7488/ds/2485.

Ethics approval and consent to participate
All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 (PPL 70/8546).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1] Institute of Evolutionary Biology, and Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh, UK. [2] Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA.

Received: 30 January 2019 Accepted: 17 May 2019
Published online: 02 July 2019

References

1. Edgar RS, Green EW, Zhao Y, van Ooijen G, Olmedo M, Qin X, et al. Peroxiredoxins are conserved markers of circadian rhythms. Nature. 2012;485:459–64.
2. Vaze KM, Sharma VK. On the adaptive significance of circadian clocks for their owners. Chronobiol Int. 2013;30:413–33.
3. Reece SE, Prior KF, Mideo N. The life and times of parasites: rhythms in strategies for within-host survival and between-host transmission. J Biol Rhythms. 2017;32:516–33.
4. Martinez-Bakker M, Helm B. The influence of biological rhythms on host-parasite interactions. Trends Ecol Evol. 2015;30:314–26.
5. Parecis-Silva PV, Nuvoloni FM, Feres RJ. Day vs. night: the importance of the circadian cycle over metacommunities and predator–prey densities. Int J Acarol. 2016;42:141–8.
6. Wamae PM, Githeko AK, Otieno GO, Kabiru EW, Duombia SO. Early biting of the *Anopheles gambiae s.s.* and its challenges to vector control using insecticide treated nets in western Kenya highlands. Acta Trop. 2015;150:136–42.
7. Cooke MK, Kahindi SC, Oriango RM, Owaga C, Ayoma E, Mabuka D, et al. 'A bite before bed': exposure to malaria vectors outside the times of net use in the highlands of western Kenya. Malar J. 2015;14:259.
8. Sougoufara S, Diedhiou SM, Doucoure S, Diagne N, Sembene PM, Harry M, et al. Biting by *Anopheles funestus* in broad daylight after use of long-lasting insecticidal nets: a new challenge to malaria elimination. Malar J. 2014;13:125.
9. Yohannes M, Boelee E. Early biting rhythm in the afro-tropical vector of malaria, *Anopheles arabiensis*, and challenges for its control in Ethiopia. Med Vet Entomol. 2012;26:103–5.
10. Moiroux N, Gomez MB, Pennetier C, Elanga E, Djenontin A, Chandre F, et al. Changes in *Anopheles funestus* biting behavior following universal coverage of long-lasting insecticidal nets in Benin. J Infect Dis. 2012;206:1622–9.
11. Russell TL, Govella NJ, Azizi S, Drakeley CJ, Kachur SP, Killeen GF. Increased proportions of outdoor feeding among residual malaria vector populations following increased use of insecticide-treated nets in rural Tanzania. Malar J. 2011;10:80.
12. Taylor B. Changes in feeding behavior of a malaria vector, *Anopheles farauti* Lav, following use of DDT as a residual spray in houses in British Solomon-Islands Protectorate. Trans R Entomol Soc Lond. 1975;127:277–92.
13. Takken W. Do insecticide-treated bednets have an effect on malaria vectors? Trop Med Int Health. 2002;7:1022–30.
14. Rund SSC, O'Donnell AJ, Gentile JE, Reece SE. Daily rhythms in mosquitoes and their consequences for malaria transmission. Insects. 2016;7:14.
15. Duffield GE, Acri DJ, George GF, Sheppard AD, Beebe NW, Ritchie SA, et al. Diel flight activity of wild-caught *Anopheles farauti* (*s.s.*) and *An. hinesorum* malaria mosquitoes from northern Queensland, Australia. Parasit Vectors. 2019;12:48.
16. Rund SSC, Hou TY, Ward SM, Collins FH, Duffield GE. Genome-wide profiling of diel and circadian gene expression in the malaria vector *Anopheles gambiae*. Proc Natl Acad Sci USA. 2011;108:E421–30.
17. Sharma VK. Adaptive significance of circadian clocks. Chronobiol Int. 2003;20:901–19.
18. Bockarie MJ, Alexander N, Bockarie F, Ibam E, Barnish G, Alpers M. The late biting habit of parous *Anopheles* mosquitoes and pre-bedtime exposure of humans to infective female mosquitoes. Trans R Soc Trop Med Hyg. 1996;90:23–5.
19. Rund SS, Bonar NA, Champion MM, Ghazi JP, Houk CM, Leming MT, et al. Daily rhythms in antennal protein and olfactory sensitivity in the malaria mosquito *Anopheles gambiae*. Sci Rep. 2013;3:2494.
20. Balmert NJ, Rund SSC, Ghazi JP, Zhou P, Duffield GE. Time-of-day specific changes in metabolic detoxification and insecticide resistance in the malaria mosquito *Anopheles gambiae*. J Insect Physiol. 2014;64:30–9.
21. Rund SS, Gentile JE, Duffield GE. Extensive circadian and light regulation of the transcriptome in the malaria mosquito *Anopheles gambiae*. BMC Genom. 2013;14:218.
22. Leming MT, Rund SS, Behura SK, Duffield GE, O'Tousa JE. A database of circadian and diel rhythmic gene expression in the yellow fever mosquito *Aedes aegypti*. BMC Genom. 2014;15:1128.
23. Graca-Souza AV, Maya-Monteiro C, Paiva-Silva GO, Braz GRC, Paes MC, Sorgine MHF, et al. Adaptations against heme toxicity in blood-feeding arthropods. Insect Biochem Mol Biol. 2006;36:322–35.
24. Williams JC, Hagedorn HH, Beyenbach KW. Dynamic changes in flow-rate and composition of urine during the post-bloodmeal diuresis in *Aedes aegypti* (L). J Comp Physiol. 1983;153:257–65.

25. DeJong RJ, Miller LM, Molina-Cruz A, Gupta L, Kumar S, Barillas-Mury C. Reactive oxygen species detoxification by catalase is a major determinant of fecundity in the mosquito *Anopheles gambiae*. Proc Natl Acad Sci USA. 2007;104:2121–6.
26. Magalhaes T, Brackney DE, Beier JC, Foy BD. Silencing an *Anopheles gambiae* catalase and sulfhydryl oxidase increases mosquito mortality after a blood meal. Arch Insect Biochem Physiol. 2008;68:134–43.
27. Oliveira JH, Goncalves RL, Lara FA, Dias FA, Gandara AC, Menna-Barreto RF, et al. Blood meal-derived heme decreases ROS levels in the midgut of *Aedes aegypti* and allows proliferation of intestinal microbiota. PLoS Pathog. 2011;7:e1001320.
28. Dong Y, Manfredini F, Dimopoulos G. Implication of the mosquito midgut microbiota in the defense against malaria parasites. PLoS Pathog. 2009;5:e1000423.
29. McDonald MJ, Rosbash M. Microarray analysis and organization of circadian gene expression in *Drosophila*. Cell. 2001;107:567–78.
30. Stone EF, Fulton BO, Ayres JS, Pham LN, Ziauddin J, Shirasu-Hiza MM. The circadian clock protein timeless regulates phagocytosis of bacteria in *Drosophila*. PLoS Pathog. 2012;8:e1002445.
31. Lee JE, Ederyl I. Circadian regulation in the ability of *Drosophila* to combat pathogenic infections. Curr Biol. 2008;18:195–9.
32. Murdock CC, Moller-Jacobs LL, Thomas MB. Complex environmental drivers of immunity and resistance in malaria mosquitoes. Proc R Soc B Biol Sci. 2013;280:20132030.
33. Schneider P, Rund SSC, Smith NL, Prior KF, O'Donnell AJ, Reece SE. Adaptive periodicity in the infectivity of malaria gametocytes to mosquitoes. Proc R Soc B Biol Sci. 2018;285:20181876.
34. Feigin RD, Dangerfield HG, Beisel WR. Circadian periodicity of blood amino-acids in normal and adrenalectomized mice. Nature. 1969;221:94–5.
35. Fernstrom JD, Larin F, Wurtman RJ. Daily variations in the concentrations of individual amino acids in rat plasma. Life Sci I. 1971;10:813–9.
36. Wurtman RJ, Rose CM, Chou C, Larin FF. Daily rhythms in the concentrations of various amino acids in human plasma. N Engl J Med. 1968;279:171–5.
37. Clark RH, Korst DR. Circadian periodicity of bone marrow mitotic activity and reticulocyte counts in rats and mice. Science. 1969;166:236–7.
38. Scheiermann C, Kunisaki Y, Frenette PS. Circadian control of the immune system. Nat Rev Immunol. 2013;13:190–8.
39. Touitou Y, Touitou C, Bogdan A, Reinberg A, Auzeby A, Beck H, et al. Differences between young and elderly subjects in seasonal and circadian variations of total plasma proteins and blood volume as reflected by hemoglobin, hematocrit, and erythrocyte counts. Clin Chem. 1986;32:801–4.
40. Korgaonkar NS, Kumar A, Yadav RS, Kabadi D, Dash AP. Mosquito biting activity on humans & detection of *Plasmodium falciparum* infection in *Anopheles stephensi* in Goa, India. Indian J Med Res. 2012;135:120–6.
41. Reisen W, Aslamkhan M. Biting rhythms of some Pakistan mosquitoes (Diptera: Culicidae). Bull Entomol Res. 1978;68:313–30.
42. Rowland M, Boersma E. Changes in the spontaneous flight activity of the mosquito *Anopheles stephensi* by parasitization with the rodent malaria *Plasmodium yoelii*. Parasitology. 1988;97:221–7.
43. Briegel H, Lea AO, Klowden MJ. Hemoglobinometry as a method for measuring blood meal sizes of mosquitoes (Diptera: Culicidae). J Med Entomol. 1979;15:235–8.
44. Rueden CT, Schindelin J, Hiner MC, DeZonia BE, Walter AE, Arena ET, et al. Image J2: ImageJ for the next generation of scientific image data. BMC Bioinform. 2017;18:529.
45. O'Donnell AJ, Schneider P, McWatters HG, Reece SE. Fitness costs of disrupting circadian rhythms in malaria parasites. Proc Biol Sci. 2011;278:2429–36.
46. Ramiro RS, Alpedrinha J, Carter L, Gardner A, Reece SE. Sex and death: the effects of innate immune factors on the sexual reproduction of malaria parasites. PLoS Pathog. 2011;7:e1001309.
47. R Development Core Team. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2018. https://www.R-project.org/.
48. Therneau TM. coxme: mixed effects Cox models. R package version 2.2-10. 2018. https://cran.r-project.org/web/packages/coxme/index.html. Accessed 3 Dec 2018.
49. Hurd H. Evolutionary drivers of parasite-induced changes in insect life-history traits from theory to underlying mechanisms. Adv Parasitol. 2009;68:85–110.
50. Vezilier J, Nicot A, Gandon S, Rivero A. *Plasmodium* infection decreases fecundity and increases survival of mosquitoes. Proc Biol Sci. 2012;279:4033–41.
51. Ahmed AM, Hurd H. Immune stimulation and malaria infection impose reproductive costs in *Anopheles gambiae* via follicular apoptosis. Microbes Infect. 2006;8:308–15.
52. Charlwood J, Graves P, Marshall TDC. Evidence for a 'memorized' home range in *Anopheles farauti* females from Papua New Guinea. Med Vet Entomol. 1988;2:101–8.
53. Charlwood JD, Graves PM. The effect of permethrin-impregnated bednets on a population of *Anopheles farauti* in coastal Papua New Guinea. Med Vet Entomol. 1987;1:319–27.
54. Hii JL, Birley MH, Kanai L, Foligeli A, Wagner J. Comparative effects of permethrin-impregnated bednets and DDT house spraying on survival rates and oviposition interval of *Anopheles farauti* No. 1 (Diptera:Culicidae) in Solomon Islands. Ann Trop Med Parasitol. 1995;89:521–9.
55. Fritz ML, Huang J, Walker ED, Bayoh MN, Vulule J, Miller JR. Ovipositional periodicity of caged *Anopheles gambiae* individuals. J Circadian Rhythms. 2008;6:2.
56. McCrae AW. Oviposition by African malaria vector mosquitoes. I. Temporal activity patterns of caged, wild-caught, freshwater *Anopheles gambiae* Giles *sensu lato*. Ann Trop Med Parasitol. 1983;77:615–25.
57. Arifin SM, Zhou Y, Davis GJ, Gentile JE, Madey GR, Collins FH. An agent-based model of the population dynamics of *Anopheles gambiae*. Malar J. 2014;13:424.
58. Lee C, Etchegaray JP, Cagampang FR, Loudon AS, Reppert SM. Post-translational mechanisms regulate the mammalian circadian clock. Cell. 2001;107:855–67.
59. Reddy AB, Karp NA, Maywood ES, Sage EA, Deery M, O'Neill JS, et al. Circadian orchestration of the hepatic proteome. Curr Biol. 2006;16:1107–15.
60. Shirasu-Hiza MM, Dionne MS, Pham LN, Ayres JS, Schneider DS. Interactions between circadian rhythm and immunity in *Drosophila melanlogaster*. Curr Biol. 2007;17:R353–5.
61. Pumpuni CB, Demaio J, Kent M, Davis JR, Beier JC. Bacterial population dynamics in three *Anopheline* species: the impact on *Plasmodium* sporogonic development. Am J Trop Med Hyg. 1996;54:214–8.
62. Uchida K. Balanced amino acid composition essential for infusion-induced egg development in the mosquito (*Culex pipiens pallens*). J Insect Physiol. 1993;39:615–21.
63. Jain SK, Hochstein P. Generation of superoxide radicals by hydrazine: its role in phenylhydrazine-induced hemolytic anemia. Biochim Biophys Acta Gen Subj. 1979;586:128–36.
64. Hurd H. Manipulation of medically important insect vectors by their parasites. Annu Rev Entomol. 2003;48:141–61.
65. Hogg JC, Hurd H. Malaria induced reduction of fecundity during the first gonotrophic cycle of *Anopheles stephensi* mosquitoes. Med Vet Entomol. 1995;9:176–80.
66. Hogg JC, Hurd H. The effects of natural *Plasmodium falciparum* infection on the fecundity and mortality of *Anopheles gambiae sl* in north east Tanzania. Parasitology. 1997;114:325–31.
67. Vezilier J, Nicot A, Gandon S, Rivero A. *Plasmodium* infection brings forward mosquito oviposition. Biol Lett. 2015;11:20140840.
68. Pigeault R, Caudron Q, Nicot A, Rivero A, Gandon S. Timing malaria transmission with mosquito fluctuations. Evol Lett. 2018;2–4:378–89.
69. Ferguson HM, Read AF. Why is the effect of malaria parasites on mosquito survival still unresolved? Trends Parasitol. 2002;18:256–61.
70. Minchella DJ, Loverde PT. A cost of increased early reproductive effort in the snail *Biomphalaria glabrata*. Am Nat. 1981;118:876–81.
71. Agnew P, Koella JC, Michalakis Y. Host life history responses to parasitism. Microbes Infect. 2000;2:891–6.
72. Garza-Hernández JA, Rodríguez-Pérez MA, Salazar MI, Russell TL, Adeleke MA, de Luna-Santillana EdJ, et al. Vectorial capacity of *Aedes aegypti* for dengue virus type 2 is reduced with co-infection of *Metarhizium anisopliae*. PLoS Negl Trop Dis. 2013;7:e2013.
73. Almeida F, Moura AS, Cardoso AF, Winter CE, Bijovsky AT, Suesdek L. Effects of *Wolbachia* on fitness of *Culex quinquefasciatus* (Diptera; Culicidae). Infect Genet Evol. 2011;11:2138–43.

74. Rono MK, Whitten MM, Oulad-Abdelghani M, Levashina EA, Marois E. The major yolk protein vitellogenin interferes with the anti-plasmodium response in the malaria mosquito *Anopheles gambiae*. PLoS Biol. 2010;8:e1000434.
75. Costa G, Gildenhard M, Eldering M, Lindquist RL, Hauser AE, Sauerwein R, et al. Non-competitive resource exploitation within-mosquito shapes evolution of malaria virulence. bioRxiv. 2017.
76. Ahmed AM, Baggott SL, Maingon R, Hurd H. The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae*. Oikos. 2002;97:371–7.

Publisher's Note

Paper #4 (2020): Host circadian clocks do not set the schedule for the within-host replication of malaria parasites

PROCEEDINGS B

royalsocietypublishing.org/journal/rspb

Research

Cite this article: O'Donnell AJ, Prior KF, Reece SE. 2020 Host circadian clocks do not set the schedule for the within-host replication of malaria parasites. *Proc. R. Soc. B* **287**: 20200347.
http://dx.doi.org/10.1098/rspb.2020.0347

Received: 17 February 2020
Accepted: 21 July 2020

Subject Category:
Ecology

Subject Areas:
evolution, ecology

Keywords:
Plasmodium, transcriptional–translational feedback loop, periodicity, circadian rhythm, clock mutant

Author for correspondence:
Aidan J. O'Donnell
e-mail: aidan.odonnell@ed.ac.uk

Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.5083700.

THE ROYAL SOCIETY PUBLISHING

Host circadian clocks do not set the schedule for the within-host replication of malaria parasites

Aidan J. O'Donnell, Kimberley F. Prior and Sarah E. Reece

Institute of Evolutionary Biology, and Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh, UK

AJO, 0000-0003-3503-094X; SER, 0000-0001-6716-6732

Circadian clocks coordinate organisms' activities with daily cycles in their environment. Parasites are subject to daily rhythms in the within-host environment, resulting from clock-control of host activities, including immune responses. Parasites also exhibit rhythms in their activities: the timing of within-host replication by malaria parasites is coordinated to host feeding rhythms. Precisely which host feeding-related rhythm(s) parasites align with and how this is achieved are unknown. Understanding rhythmic replication in malaria parasites matters because it underpins disease symptoms and fuels transmission investment. We test if rhythmicity in parasite replication is coordinated with the host's feeding-related rhythms and/or rhythms driven by the host's canonical circadian clock. We find that parasite rhythms coordinate with the time of day that hosts feed in both wild-type and clock-mutant hosts, whereas parasite rhythms become dampened in clock-mutant hosts that eat continuously. Our results hold whether infections are initiated with synchronous or with desynchronized parasites. We conclude that malaria parasite replication is coordinated to rhythmic host processes that are independent of the core-clock proteins PERIOD 1 and 2; most likely, a periodic nutrient made available when the host digests food. Thus, novel interventions could disrupt parasite rhythms to reduce their fitness, without interference by host clock-controlled homeostasis.

1. Introduction

Biological rhythms are ubiquitous and allow organisms to maximize fitness by synchronizing behaviours, physiologies and cellular processes with periodicity in their environment. The value of coordinating with daily cycles in light/dark (LD) and temperature in the abiotic environment has long been appreciated, and the importance for parasites of coordinating with rhythms experienced inside hosts and vectors (i.e. the biotic environment) is gaining recognition [1–3]. For example, circadian rhythms in virulence enables the fungal pathogen *Botrytis cinerea* to cope with rhythmic immune defences in plant hosts [4,5], circadian control of macrophage migration provides incoming *Leishmania major* parasites with more host cells to invade at dusk than dawn [6] and host clocks control the ability of herpes and hepatitis viruses to invade cells and to replicate within them [7,8].

Malaria (*Plasmodium*) parasites exhibit periodicity in their development during cycles of asexual replication in red blood cells (the intra-erythrocytic development cycle; IDC). No known clock genes have been identified in *Plasmodium* genomes, but their gene expression patterns display some hallmarks of an endogenous clock [9,10]. Explaining how and why malaria parasites complete their IDC according to a particular schedule matters because cycles of asexual replication are responsible for the severity of malaria symptoms and fuel the production of transmission forms, and coordination with

the rhythms of both host and vector enhances parasite fitness [11–15]. Thus, insight into the IDC schedule may suggest novel interventions to disrupt parasite replication. Indeed, many antimalarial drugs have increased efficacy against specific IDC stages [16] and IDC stage-specific dormancy may facilitate parasite survival during antimalarial drug treatment [17].

The IDC lasts 24 h (or multiples of 24 h, depending on the species) and is characterized by progression through distinct developmental stages at particular times of day. For example, the timing of *Plasmodium chabaudi's* IDC transitions coincide with the time of day that murine hosts are provided with food [18,19]. Specifically, parasites remain in early IDC stages when hosts are fasting and complete the IDC at the end of the feeding phase. The foundation for explaining both why the IDC schedule benefits parasites and how it is controlled lies in discovering which of the myriad of host rhythms associated with the time of day that hosts feed also associate with the timing of the IDC schedule. Here, we use the rodent malaria parasite *P. chabaudi* to test whether the IDC schedule is coordinated with a host rhythm(s) that is driven by—or is independent of—the transcription–translation feedback loop (TTFL) that forms a major part of the host's circadian clock mechanism. The mammalian circadian clock operates via a core TTFL (which we hereafter call the core-TTFL clock) involving dimeric proteins that promote the expression of other clock proteins as well as the inhibition of themselves [20]. The feedback and degradation of these proteins forms an oscillator that is entrained via external daily stimuli (Zeitgeber, usually light) to keep the clock precisely tuned to environmental periodicity.

Core-TTFL clock-controlled processes undertaken by the host include many metabolic pathways relevant to IDC progression. For example, CLOCK and BMAL1 are involved in regulating blood glucose levels [21,22] and melatonin release, which are both implicated in IDC completion [18,19,23]. Alternatively, the IDC schedule could simply be aligned to the appearance of nutrients/metabolites made available in the blood as a direct consequence of food digestion (i.e. via processes not reliant on the host TTFL clock). Core-TTFL clock-controlled, and TTFL-independent products of digestion, could act in several non-mutually exclusive ways on the IDC, including: (i) impacting directly on IDC progression by providing essential resources for different IDC stages at different times of day, (ii) providing time-of-day information to the parasite to modulate its rate of development to maximize acquisition of such resources and (iii) act as a proxy for the timing (phase) of another important rhythmic factor that the parasite must coordinate with. Most of these scenarios, and most evidence to date [10–13,19,24], suggests the parasite possesses an ability to keep time.

To probe how core-TTFL clock-controlled host rhythms and host-feeding-related rhythms influence the IDC schedule, we apply time-restricted feeding (TRF) protocols to wild-type (WT) mice and clock-disrupted *Per1–Per2* double knockout mice (*Per1/2*-null) and compare the consequences for the IDC schedule of *P. chabaudi* infections initiated with either synchronous or desynchronized parasites. We hypothesize that if the IDC is scheduled according to a host-feeding-related rhythm alone, IDC completion will coincide with host feeding in WT and in *Per1/2*-null mice with a feeding rhythm (TRF), but that parasites become (or remain) desynchronized in *Per1/2*-null mice allowed to feed continuously. By contrast, if feeding rhythms influence the IDC schedule via host TTFL-clock-controlled processes, parasites will only become (or remain) synchronous in WT mice, because they have both clocks and a feeding rhythm. We also test whether infection of TTFL-clock-disrupted mice has fitness consequences for both parasites and hosts. The mammalian TTFL-clock controls many aspects of rhythmicity in immunity [25], including the ability of leucocytes to migrate to the tissues [26] and the ability of macrophages to release cytokines [27]. Furthermore, rodents without functioning *Per2* lack IFN-γ mRNA cycling in the spleen (a key organ for malaria parasite clearance) and have decreased levels of pro-inflammatory cytokines in blood serum [28]. Thus, we predict that parasites will achieve higher densities, and hosts experience more severe disease, in *Per1/2*-null compared to WT mice.

2. Methodology

To test if rhythmicity in parasite replication is coordinated with the host's feeding-related rhythms and/or rhythms driven by the host's canonical circadian clock, we performed two experiments. First, we initiated infections with desynchronized parasites to test whether a host feeding rhythm alone is sufficient to restore synchrony and timing in the IDC (figure 1*a*). Second, we tested whether the loss of rhythmic host feeding leads to desynchronization of the IDC in infections initiated with synchronized parasites (figure 1*b*).

(a) Parasites and hosts

Hosts were either WT C57BL/6 J strain or *Per1/2*-null clock-disrupted mice previously backcrossed onto a C57BL/6 J background for over 10 generations. *Per1/2*-null mice (kindly donated by Michael Hastings, MRC Laboratory of Molecular Biology, Cambridge, UK) derived from JAX strains #010831 (129S-Per1^{tm1Drw}/J) and #010492 (129S-Per1^{tm1Drw}/J) generated by David Weaver (UMass Medical School, MA, USA) have an impaired core-TTFL clock and exhibit no known circadian rhythms in physiology and behaviour. For example, their locomotor activity is arrhythmic when placed in constant conditions, such as constant darkness [29,30]. We housed all experimental WT and *Per1/2*-null mice (8–10 weeks old) at 21°C in DD (continuous darkness) 'free-running' conditions with constant dim red LED light for three weeks prior to, and throughout the duration of infections. This allowed sufficient time for the erosion of residual (ultradian or unconsolidated) rhythms that can persist when clock-disrupted mice enter DD conditions [29]. Note, we housed donor mice in LD cycle conditions to generate synchronous parasites for the initiation of experimental infections. As the period (the time taken for a rhythm to complete one full cycle) of our WT mice is very close to 24 h (23.8–23.9 h; electronic supplementary material) when placed in DD, these mice exhibit rhythms very similar to the LD conditions they were raised in. Therefore, we define subjective day (rest phase) for WT mice as 07.00–19.00 GMT and subjective night (active phase) as 19.00–07.00 GMT.

We fed all mice on a standard RM3 pelleted diet (801700, SDS, UK) with unrestricted access to drinking water supplemented with 0.05% *para*-aminobenzoic acid (to supplement parasite growth, as is routine for this model system). All mice were acclimatized to their feeding treatments (see below) for three weeks before and throughout infections and housed individually to avoid any influence of conspecific cage-mates on their rhythms. On Day 0, we infected each mouse with 5×10^6 *P. chabaudi* (clone DK) parasitized red blood cells administered

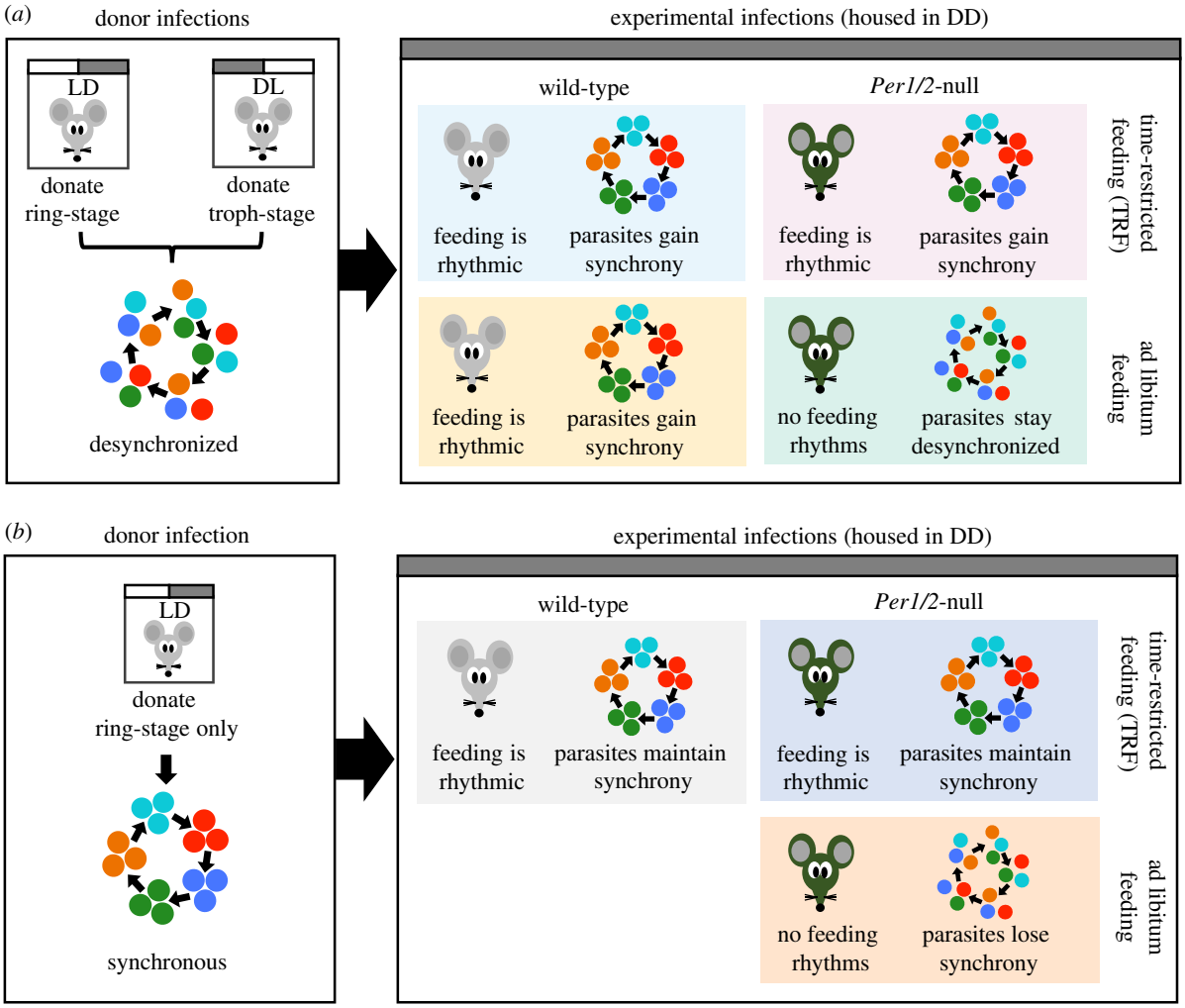

Figure 1. Experimental designs and predictions. Donor infections from mice housed in LD and/or DL were used to generate a desynchronized (*a*; ring stage + trophozoite stage parasites) and synchronous (*b*; ring stage parasites only) inocula for initiating experimental infections. WT or *Per1/2*-null clock-disrupted mice were given constant access to food (ad libitum) or fed on a TRF schedule in which food access was restricted to only 10 h d^{-1}. These mice were used as hosts for experimental infections and sampled every 4 h for 32 h on Day 5 and 6 PI. We predicted that desynchronized infections will become synchronous in mice in which feeding is rhythmic (both WT groups and *Per1/2*-null TRF) but will remain desynchronized in the ad libitum fed *Per1/2*-null mice due to a lack of host feeding rhythms. Furthermore, we expected the timing of parasites that become synchronous will match host feeding rhythms. Thus, parasites in WT ad libitum fed mice will follow the opposite schedule to parasites in WT TRF and *Per1/2*-null TRF mice (*a*). For infections initiated with synchronous parasites, we predicted that parasites will maintain synchrony in WT and *Per1/2*-null TRF groups and that the IDC schedule changes to match the timing of host feeding, but that synchrony will decay in parasites in ad libitum fed *Per1/2*-null mice, which lack feeding rhythms (*b*). (Online version in colour.)

via intravenous injection. All procedures complied with the UK Animals (Scientific Procedures) Act 1986 (PPL 70/8546).

(b) Experimental designs

WT mice and *Per1/2*-null mice experienced a TRF schedule (fed for 10 h d^{-1}) or had continuous access to food ad libitum. The TRF mice remained in their cages during food provision and removal, to minimize disturbance, and food was provided/removed by changing the lid (which held the food) and sweeping the cage for stray pellets at the times of removal.

Despite continuous access to food, WT mice followed their normal free-running rhythms and fed primarily in their subjective night (19.00–07.00 GMT). Whereas, WT TRF mice fed only in their subjective day (09.00–19.00 GMT), causing temporal misalignment between rhythms controlled by the suprachiasmatic nucleus (SCN) and peripheral rhythms [19]. *Per1/2*-null TRF mice fed during the day (09.00–19.00 GMT) only experienced rhythms resulting from a set daily period of feeding (electronic supplementary material), whereas ad libitum *Per1/2*-null mice were arrhythmic (electronic supplementary material) due to continuous feeding. TRF feeding regimes do not cause caloric restriction because mice are given unrestricted access to food during their daily feeding window.

(c) Experiment 1: can desynchronized parasites restore the intra-erythrocytic development cycle schedule in hosts with a feeding rhythm?

We generated four treatment groups of $n = 5$ mice (figure 1*a*): (i) WT ad libitum fed mice that naturally feed during subjective night; (ii) WT TRF mice fed only during subjective day; (iii) *Per1/2*-null mice fed ad libitum; and (iv) *Per1/2*-null TRF mice fed during the day. We initiated infections in all mice with a population of desynchronized parasites at 08.30 GMT by using an inoculum of a 50 : 50 mix of parasites 12 h apart in their IDC. Specifically, we mixed ring stages (donated from donors in a 12 : 12 LD cycle) and late trophozoite stages (donated from dark : light (DL) donors) (figure 1*a*).

(d) Experiment 2: do parasites lose intra-erythrocytic development cycle synchrony in the absence of a host feeding rhythm?

We generated three groups of $n = 5$ mice (figure 1*b*): (i) WT TRF mice fed during subjective day; (ii) *Per1/2*-null TRF mice

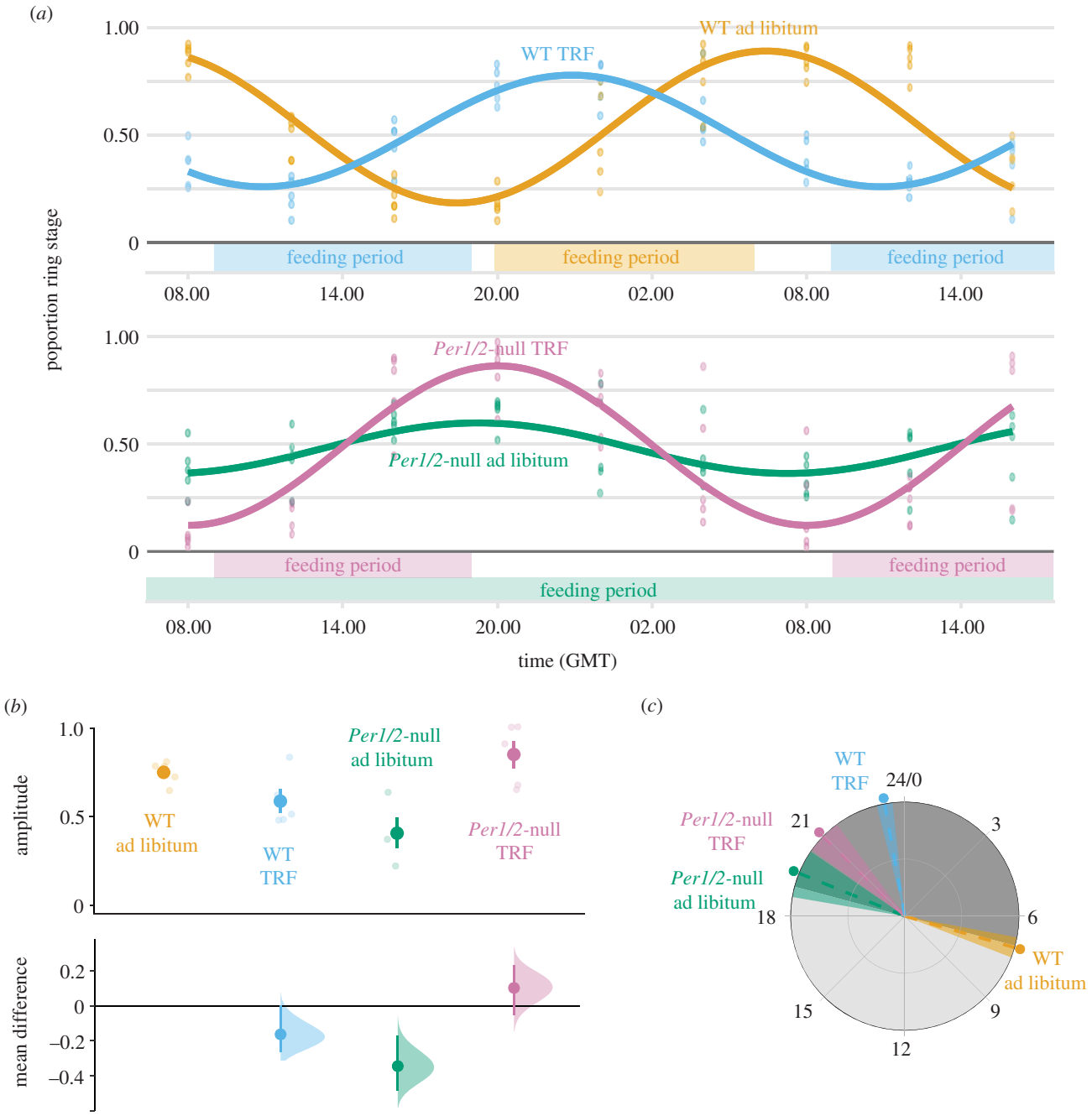

Figure 2. The IDC of desynchronized parasites becomes coordinated to host feeding-associated rhythms. Population cosinor model fits and data points from each individual infection (Day 5–6 PI) (*a*). Amplitude (*b*) and phase in hours (GMT) (*c*) were calculated from cosinor model fits from each individual mouse (lighter points) and then summarized as a mean ± s.e.m., points with error bars in (*b*), and circular mean ± s.d. point with dashed line and shading in (*c*). For amplitude (*b*), effect sizes relative to the 'WT ad libitum' group are plotted on the lower axes as a bootstrap sampling distribution (mean difference ± 95% CI depicted as a point with error bars). For all parts, infections in WT hosts are coloured orange and blue, and infections in *Per1/2*-null mice are coloured green and purple ($n = 5$ for the WT and TRF groups, $n = 4$ for *Per1/2*-null ad libitum group). TRF indicates 'time-restricted feeding' with food only available for 10 h each day (feeding period indicated above *x* axis in (*a*)). Grey shading in (*c*) represents active (dark shading; 19.00–07.00) and rest (light shading; 07.00–19.00) periods relative to WT mice in DD. (Online version in colour.)

fed during the day; and (iii) *Per1/2*-null with continuous (ad libitum) access to food. We infected all mice with a population of synchronous ring stage parasites early in the feeding period (which is 12 h out of phase to when rings stages peak in control infections (figure 1*b*)). Generating a mismatch between incoming parasites and the recipient host's feeding rhythm tests whether the IDC becomes rescheduled to match the feeding rhythm in the TRF groups. This avoids an outcome of the IDC being constrained and unable to change schedule obscuring the importance of a host-feeding rhythm, following the design in Prior *et al*. [19].

(e) Sampling and data collection

We sampled all experimental mice at 4-hourly intervals for 32 h beginning at 08.00 (GMT) Day 5 to 16.00 Day 6 post-infection (PI). Previous work [19] revealed that synchronous parasites in infections initially mismatched to the host's feeding rhythm by 12 h (as we do in Experiment 2) exhibit a rescheduled IDC within 4 days. This phenomenon is verified here: the IDC became rescheduled in the WT TRF mice fed during subjective day (figures 2*a* and 3*a*). At each sampling point, we collected blood from the tail vein and quantified each IDC stage from

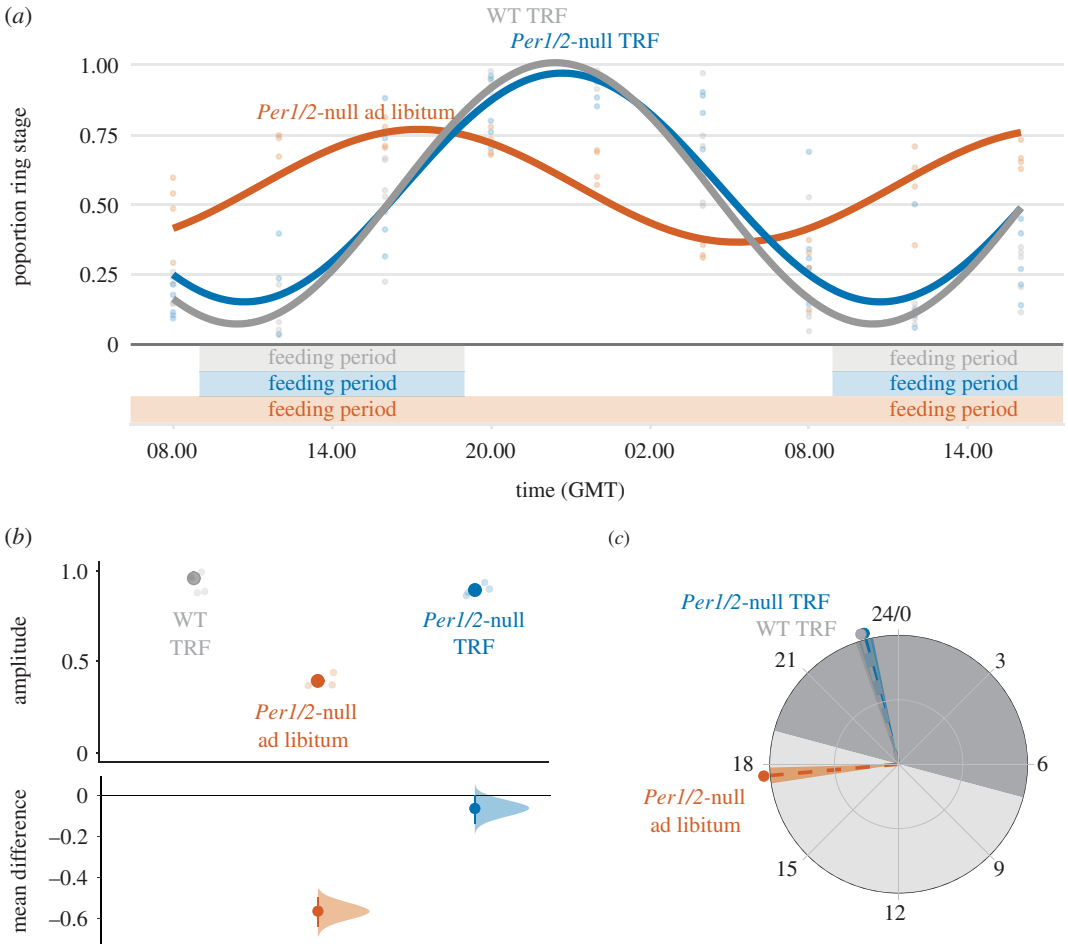

Figure 3. IDC synchrony is reduced in hosts without feeding-associated rhythms. Population cosinor model fits and data points from each individual infection (Day 5–6 PI) (*a*). Amplitude (*b*) and phase in hours (GMT) (*c*) were calculated from cosinor model fits from each individual mouse (lighter points) and then summarized as a mean ± s.e.m., points with error bars in (*b*) and circular mean ± s.d. point with dashed line and shading in (*c*). For amplitude (*b*), effect sizes relative to the 'WT TRF' group are plotted on the lower axes as a bootstrap sampling distribution (mean difference ± 95% CI depicted as a point with error bars). For all parts, infections in WT are coloured grey, and infections in *Per1/2*-null mice are coloured orange and blue ($n = 5$ for WT, $n = 4$ for TRF and $n = 3$ for *Per1/2*-null ad libitum group). TRF indicates 'time-restricted feeding' with food only available for 10 h each day (feeding period indicated above *x* axis in (*a*)). Grey shading in (*c*) represents active (dark shading; 19.00–07.00) and rest (light shading; 07.00–19.00) periods relative to WT mice in DD. (Online version in colour.)

thin blood smears, as is standard for measuring the IDC schedule [10–13,19,24]. Specifically, we characterized stages by morphology, based on parasite size, the size and number of nuclei and the appearance of haemozoin (as per [19,31]). We measured red blood cell (RBC) densities at each sampling time by flow cytometry (Z2 Coulter Counter, Beckman Coulter), and mouse weights on Day 2 PI and Day 6 PI at 16.00 GMT. All procedures were carried out in dim red LED light. Before infection, we characterized rhythms in locomotor activity (movement around the cage) and internal body temperature for all host genotype and feeding regime combinations (electronic supplementary material), and tested whether locomotor activity is a proxy for feeding events (electronic supplementary material). Data were analysed using Clocklab, CircWave, JTK_CYCLE and R (see electronic supplementary material, for details).

3. Results

(a) Assumptions of the experimental designs

We first verified that WT mice exhibit rhythms in locomotor activity and body temperature, and also confirmed arrhythmic activity of *Per1/2*-null ad libitum fed mice (electronic supplementary material, Methods, and figures S1–S3). We then verified that locomotor activity can be used as a proxy for feeding rhythms in *Per1/2*-null TRF mice (electronic supplementary material, Methods and figure S4).

(b) Experiment 1: can desynchronized parasites restore the intra-erythrocytic development cycle schedule in hosts with a feeding rhythm?

We compared IDC rhythms in terms of synchronicity (amplitude) and timing (phase) of the proportion of parasites at ring stage (a morphologically distinct 'marker' stage after which all other parasite stages follow in a predictable manner) [19]. We do not estimate period due to the short sampling window. By Day 5–6 PI, the IDC of parasites in all WT mice and *Per1/2*-null TRF mice had become synchronized and scheduled to coincide with host feeding rhythms (figure 2*a*). Amplitude differed significantly between groups (figure 2*b*; genotype : feeding_regime: $F_{1,15} = 20.54$, $p < 0.001$). Specifically, parasites in *Per1/2*-null TRF mice had the highest amplitudes (mean ± s.e.m.: 0.85 ± 0.08) followed by WT ad libitum infections (0.75 ± 0.03), and then WT TRF infections (0.59 ± 0.07), with *Per1/2*-null ad libitum infections (0.41 ± 0.09) exhibiting approximately half the amplitude of parasites in hosts with feeding rhythms. Concomitantly, the timing of

peak ring stage proportion was explained by a host genotype : feeding regime interaction (figure 2*c*; electronic supplementary material, table S3): peaking in WT mice at the end of the host's feeding window (circular mean ± s.d. (hours GMT): WT ad libitum = 7.06 ± 0.35), and within 1–2 h of the end of the host's feeding window in TRF mice (WT TRF = 23.32 ± 0.27, *Per1*/2-null TRF = 20.96 ± 0.61). Despite the severely dampened rhythm, ring stages in ad libitum fed *Per1*/2-null mice peaked at 19.47 GMT (±0.83). See electronic supplementary material, for CircWave model fits, results from JTK_CYCLE, and mean effect sizes.

We also assessed whether anaemia and parasite performance varied between WT and *Per1*/2-null mice. Neither host genotype, feeding regime or their interaction significantly affected RBC loss (genotype : feeding_regime: $F_{1,16} = 0.27$, $p = 0.61$; host genotype: $F_{1,17} = 1.95$, $p = 0.18$; feeding regime: $F_{1,18} = 0.16$, $p = 0.70$), with hosts losing an average of 2.50 ± 0.18 s.e.m. × 10^9 ml^{-1} RBCs during the sampling period (electronic supplementary material, figure S5a and table S4). By contrast, host genotype had a significant effect on maximum parasite density ($F_{1,18} = 12.86$, $p = 0.002$) in which parasites infecting WT hosts achieved maximum densities approximately 40% higher than parasites infecting *Per1*/2-null mice (mean ± s.e.m. × 10^9 ml^{-1}: WT = 1.69 ± 0.08, *Per1*/2-null = 1.22 ± 0.10; electronic supplementary material, figure S5b and table S4). Neither feeding regime ($F_{(1,17)} = 0.24$, $p = 0.63$) nor its interaction with host genotype ($F_{1,16} = 0.36$, $p = 0.56$) had an effect on maximum parasite density.

(c) Experiment 2: do parasites lose intra-erythrocytic development cycle synchrony in the absence of a host feeding rhythm?

By Day 5–6 PI, the IDC of parasites in TRF mice had rescheduled to coincide with host feeding rhythms (figure 3*a*) verifying that sufficient time had been allowed for the IDC schedule to respond to the different perturbations of host TTFL-clock and feeding rhythms across treatment groups. IDC synchrony differed significantly between treatment groups (figure 3*b*; $F_{2,9} = 91.40$, $p < 0.001$), remaining high in TRF mice and dampening in *Per1*/2-null ad libitum mice. Specifically, ring stage amplitudes in TRF mice (mean ± s.e.m.: WT TRF = 0.96 ± 0.04, *Per1*/2-null TRF = 0.90 ± 0.02) were more than 50% higher than amplitudes for ring stages in *Per1*/2-null ad libitum mice (0.39 ± 0.02). In addition, the timing of the IDC varied across treatment groups (figure 3*c*; electronic supplementary material, table S3). Ring stages in TRF mice peaked 4 h after their host's feeding window (circular mean ± s.d. (hours GMT); WT TRF = 22.92 ± 0.22, *Per1*/2-null TRF = 23.01 ± 0.22), whereas ring stages with dampened rhythms in *Per1*/2-null ad libitum mice peaked 8 h earlier than in TRF groups (17.66 ± 0.24). See electronic supplementary material, for CircWave model fits, results from JTK_CYCLE, and mean effect sizes.

In contrast with infections initiated with desynchronized parasites, RBC loss varied across treatment groups of infections initiated with synchronous parasites ($F_{2,11} = 23.62$, $p < 0.001$; electronic supplementary material, figure S6a and table S4). WT TRF mice lost the most RBCs (30–57% more than both groups of *Per1*/2-null mice), and ad libitum fed *Per1*/2-null mice lost 20% more RBCs than their *Per1*/2-null TRF counterparts (mean RBC loss ± s.e.m. × 10^9 ml^{-1}: WT TRF = 3.57 ± 0.13, *Per1*/2-null TRF = 2.28 ± 0.15, *Per1*/2-null ad libitum = 2.73 ± 0.13). As we found for infections initiated with desynchronized parasites, maximum parasite density of infections initiated with synchronous parasites also varied across treatment groups ($F_{2,11} = 4.40$, $p = 0.04$). Parasites in WT TRF hosts achieved maximum parasite densities 24% higher than parasites in both groups of *Per1*/2-null mice (mean max. density ± s.e.m. × 10^9 ml^{-1}: WT TRF = 1.89 ± 0.11, *Per1*/2-null = 1.51 ± 0.07; electronic supplementary material, figure S6b and table S4).

4. Discussion

Our results demonstrate that timing and synchrony of the malaria parasite *P. chabaudi's* IDC is not dependent on rhythms driven by the core-TTFL clock of hosts, and that parasites establish an IDC schedule in hosts with only rhythms associated with feeding. Our first experiment revealed that parasites within infections initiated with desynchronized parasites became synchronized in hosts with a feeding rhythm, and these infections exhibited a similar timing (reaching an average peak ring stage proportion of 86% within an hour after the feeding window ends). Furthermore, in ad libitum fed clock-disrupted hosts, which feed in many small irregular bouts across each 24 h period, the IDC remained desynchronized (ring stage proportion remaining at around 50% across all sampling points). Consistent with these phenomena, our second experiment revealed that the IDC of parasites in infections initiated with synchronous parasites remained synchronous and became coordinated to the timing of host feeding but only in hosts with feeding rhythms. Whereas in ad libitum fed clock-disrupted mice, the IDC rhythm became dampened (peak in ring stages dropping from approx. 100% to approx. 75%). Put another way, both experiments show that an IDC schedule emerges in hosts with a feeding rhythm independently of the host's core-TTFL clock, and the IDC rhythm is dampened in hosts without a feeding rhythm. We expect our findings to generalize across strains, given the similarities in the IDC schedules observed in this study and in Hirako *et al.* [18] and Prior *et al.* [31] which used different strains of *P. chabaudi*.

While the IDC rhythm of synchronous parasites inoculated into ad libitum fed clock-disrupted mice became dampened, it did not become fully desynchronized. There are two non-mutually exclusive reasons for this. First, there are likely to be developmental constraints acting on the duration of each IDC stage and the overall IDC length, independent of the influence of any host scheduling forces or parasite time-keeping abilities. If the minimum and maximum duration of the IDC is close to 24 h, or stage durations are similarly constrained, natural variation in IDC duration will take more cycles to fully erode synchrony than allowed in our experiment [32]. Determining how many cycles it will take a population of parasites to fully desynchronize is complex because the rate will be obscured by changes in density [33]. Thus, without sophisticated modelling that accounts for infection dynamics, it is difficult to determine whether desynchronization of the IDC, when hosts are in constant conditions, is due to a free-running oscillator belonging to the parasite [9]. Second, even in completely asynchronous infections, the expansion of parasite number due to each asexual stage replacing itself with multiple progeny can

generate the illusion of strong synchrony [33]. We also observed different degrees of synchrony across infections in which parasites became or remained synchronous. This could be due to a combined influence of multiple host rhythms on the IDC schedule, including minor contributions from non-feeding rhythms. For example, the decoupling of SCN-driven and peripheral rhythms in the WT TRF mice could impose conflicting timing information on the IDC compared to parasites in WT ad libitum fed mice who experienced better-aligned host rhythms. Furthermore, such conflict would not occur in the TRF clock-disrupted mice, in which only food-associated rhythms are present.

Why should a rhythm(s) associated with host feeding set the schedule for the parasite IDC? Food digestion provides glucose, for example, to the host and parasite, and blood glucose concentration follows a daily rhythm in hosts mounting immune responses [18]. Glucose tolerance changes during the day in a circadian manner and behavioural factors, such as host activity, feeding and fasting, strongly affect glucose metabolism. However, glucose regulation is a complex and tightly controlled process, achieved via the antagonistic effects of the hormones insulin and glucagon, and involves the contribution of several different organs (liver, pancreas) to dampen perturbations due to feeding and fasting. This makes it difficult to separate the contributions of host TTFL-clock-dependent and -independent processes on daily rhythms in glucose concentration [34]. In addition to glucose, IDC completion relies on other nutrients from the host's food, including amino acids essential for protein synthesis [35], purines (in particular hypoxanthine) for nucleic acid synthesis and lysophosphatidylcholine, for various processes such as cell membrane production [36]. Metabolomics-around-the-clock studies may help determine which rhythm(s) related to host feeding influences the IDC schedule.

Our results suggest that a product of food digestion schedules the IDC, supporting those of Prior *et al.* [19] and Hirako *et al.* [18], yet—at first glance—apparently contradicting two experiments, relating to food intake and infection of TTFL-clock disrupted mice, respectively, in Rijo-Ferreira *et al.* [10]. First, the food intake experiment in Rijo-Ferreira *et al.* [10] aimed to test if the act of eating itself schedules the IDC. They infected WT mice housed in LD cycles, thus despite food provision being spread evenly throughout the day and night, these hosts retained their nocturnal lifestyle, including undergoing the bulk of metabolism at night. In keeping with nocturnal rhythms, hosts whose food was spread evenly throughout the day and night seem to eat more pellets at night (higher night-time versus day-time mean in fig. 3C in Rijo-Ferreira *et al.* [10]). Second, like our design, Rijo-Ferreira *et al.* [10] gave TTFL-clock disrupted mice (Cry1/Cry2 null) food ad libitum and housed them in constant darkness, yet they found IDC rhythms (in infections started with synchronous parasites) remained strong. Rijo-Ferreira *et al.*'s [10] mice were kept in LD cycles until the point of infection which may allow residual rhythms generated by masking to persist for the first few days of infection. Indeed, when these TTFL-clock disrupted hosts are housed in constant darkness for a week before infection, IDC rhythms do become dampened (fig. 4L in Rijo-Ferreira *et al.*) [10].

While our experiments rule out a role for host TTFL-clock-driven rhythms in the IDC schedule, many host processes are rhythmic in clock-disrupted mice. For example, liver genes in clock-disrupted mice express rhythmicity simply as a result of TRF protocols [37–40]. It is unclear to what extent this is due to a host endogenous oscillator independent of canonical TTFL-clock genes, such as that driving food anticipatory activity (FAA) [41–43]. Our study was not designed to quantify FAA, but nonetheless, our TRF fed clock-disrupted mice do exhibit behaviour consistent with FAA. Specifically, we observe a rise in body temperature and activity in anticipation of the 09.00 GMT feeding events (1–2 h before feeding; electronic supplementary material, figure S3). The precise mechanisms underpinning FAA rhythms are not fully understood, but they are thought to be independent of light-entrained oscillators and may use inputs such as levels of the 'hunger hormone' ghrelin, or insulin for entrainment [44]. Thus, it remains possible that the IDC schedule is aligned to the downstream consequences of such an oscillator. Similarly, daily oxidation–reduction rhythms exist within mammalian RBCs independent of a TTFL clock [45], are evolutionarily conserved [46] and may be linked to cellular flux in magnesium ions [47]. Recent work suggests that these rhythms are unlikely to impact on development during the IDC [9,24], but this is yet to be formally tested.

For small mammals, body temperature rhythms are influenced by a combination of the TTFL-clock, metabolism and locomotor activity. Almost all mice in which the IDC became or remained highly synchronous exhibited temperature rhythms to some extent (electronic supplementary material, figures S1–S3). Temperature rhythms can entrain host cells (including RBCs) and other parasites (e.g. *Trypanosoma brucei*) [48], and malaria parasites do respond to temperature change (e.g. to initiate gametogenesis when taken up by ectothermic mosquitoes) [49,50]. However, it is unlikely that the IDC schedule is aligned to a temperature rhythm. For example, Prior *et al.* [19] reveal inverted IDC rhythms in day- and night-fed mice, but host temperature rhythms are not inverted. More generally, temperature could only provide time-of-day resolution of 12 h and the IDC schedule is more precise than, for example, completion 'occurring at any point during the host's warm phase'. Alternatively, parasites may use a sharp change in temperature to determine time of day; however, multiple sharp temperature rises and drops exist throughout the day (electronic supplementary material, figure S3b) which suggest temperate change is also an unreliable indicator for time of day. A solution to this could be that only certain IDC stages are sensitive to temperature (so, misleading temperature changes are ignored), but if this were the case then parasites in infections mismatched to host rhythms (the WT and *Per1/2*-null TRF in both experiments presented here and in [11–13,19]) would not become rescheduled.

We also used our data to test whether parasite performance is enhanced in clock-disrupted hosts, potentially due to lack of regulation/coordination of TTFL-clock-controlled immune responses [25,28]. However, we find that the maximum parasite density is approximately 25–40% (across both experiments) lower in infections of clock-disrupted compared to WT hosts. Clock-disruption might reduce the ability of hosts to process and metabolize food efficiently, making these hosts a poorer resource for parasites. For example, PER1 and PER2 have a regulatory role in the circadian control of haem synthesis [51], with haemoglobin catabolism providing a primary source of amino acids for parasites, and loss of PER2 is implicated in making RBCs more susceptible to

oxidative stress, decreasing levels of ATP and shortening RBC lifespan [52]. Further, clock-disruption affects host nutrition via an interplay with microbiota [53]. Parasite performance is linked to host nutrition because caloric restriction leads to reduced parasite densities [54]. However, if either clock disruption and/or our time-restricted-feeding regime caused caloric restriction, we would expect this to manifest as clock-disrupted mice—especially in the TRF group—as having the lowest weights or the greatest weight loss. By contrast, clock-disrupted TRF mice were the heaviest in experiment 1 and clock-disrupted ad libitum fed mice lost the most weight in Experiment 2 (electronic supplementary material, table S5). Another, non-mutually exclusive, possibility is that the IDC becomes rescheduled faster in WT mice, and the faster that parasites can reschedule, the lower the fitness costs of being uncoordinated with the host's feeding rhythm. However, that parasite performance does not differ between infections remaining/becoming desynchronized versus synchronous within the same type of host (i.e. *Per1/2*-null) suggests either that there are no major costs to parasites of being desynchronized or that it is advantageous for them to match the degree of rhythmicity of their host. While the costs of virulence, as measured by weight loss, do not appear to differ between WT and clock-disrupted hosts, the findings for RBC loss are more complicated (and do not clearly mirror maximum parasite densities). No significant difference between feeding regimes or host genotypes was detected when infections were initiated with desynchronized parasites. But, in infections initiated with synchronous parasites, WT hosts became the most anaemic and clock-disrupted hosts with a feeding rhythm lost the fewest RBCs. Further work is needed to establish whether a loss of canonical clock regulation affects the ability of hosts to control or tolerate parasites.

5. Conclusion

The schedule (timing and synchrony) of the malaria parasite's IDC is not reliant on a functioning host core-TTFL clock. The speed with which the IDC schedule changes, its precision and the modest initial loss of parasite number involved in rescheduling [12] along with parasite control of IDC duration [24] suggest the parasite is actively aligning certain developmental stages with host feeding rhythms to take advantage of periodicity in a resource(s) it must acquire from the host's food processing. Recent studies suggest that IDC rhythms display a hallmark of a circadian clock [9,10], but other criteria (temperature compensation and entrainment) are yet to be met. Whatever the parasites' method of time-keeping, our data suggest it uses a signal stemming from the host's processing of food as a Zeitgeber or timing cue. Our data also highlight a complex interplay between host rhythms, features of the IDC schedule, parasite fitness (as approximated by maximum density) and disease severity. Unravelling these complexities may reveal interventions that minimize disease severity and improve recovery, while reducing parasite fitness.

Ethics. All procedures complied with the UK Animals (Scientific Procedures) Act 1986 (PPL 70/8546).

Data accessibility. Datasets are available in the Edinburgh DataShare repository (doi:10.7488/ds/2622).

Authors' contributions. A.J.O'D. and S.E.R. conceived the study, A.J.O'D. and K.F.P. carried out the experiments, A.J.O'D. analysed the data and all authors wrote the manuscript.

Competing interests. We declare we have no competing interests.

Funding. This work was supported by Wellcome (grant nos. 202769/Z/16/Z; 204511/Z/16/Z), the Royal Society (grant nos. UF110155; NF140517) and the Human Frontier Science Program (grant no. RGP0046/2013).

Acknowledgements. We thank Ronnie Mooney and Giles K. P. Barra for technical assistance.

References

1. Martinez-Bakker M, Helm B. 2015 The influence of biological rhythms on host–parasite interactions. *Trends Ecol. Evol.* **30**, 314–326. (doi:10.1016/j.tree.2015.03.012)
2. Reece SE, Prior KF, Mideo N. 2017 The life and times of parasites: rhythms in strategies for within-host survival and between-host transmission. *J. Biol. Rhythms* **32**, 516–533. (doi:10.1177/0748730417718904)
3. Westwood ML, O'Donnell AJ, de Bekker C, Lively CM, Zuk M, Reece SE. 2019 The evolutionary ecology of circadian rhythms in infection. *Nat. Ecol. Evol.* **3**, 552–560. (doi:10.1038/s41559-019-0831-4)
4. Hevia MA, Canessa P, Müller-Esparza H, Larrondo LF. 2015 A circadian oscillator in the fungus *Botrytis cinerea* regulates virulence when infecting *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* **112**, 8744–8749. (doi:10.1073/pnas.1508432112)
5. Roden LC, Ingle RA. 2009 Lights, rhythms, infection: the role of light and the circadian clock in determining the outcome of plant–pathogen interactions. *Plant Cell* **21**, 2546–2552. (doi:10.1105/tpc.109.069922)
6. Kiessling S, Dubeau-Laramée G, Ohm H, Labrecque N, Olivier M, Cermakian N. 2017 The circadian clock in immune cells controls the magnitude of *Leishmania* parasite infection. *Sci. Rep.* **7**, 10892. (doi:10.1038/s41598-017-11297-8)
7. Edgar RS, Stangherlin A, Nagy AD, Nicoll MP, Efstathiou S, O'Neill JS, Reddy AB. 2016 Cell autonomous regulation of herpes and influenza virus infection by the circadian clock. *Proc. Natl Acad. Sci. USA* **113**, 10 085–10 090. (doi:10.1073/pnas.1601895113)
8. Zhuang X *et al.* 2019 The circadian clock components BMAL1 and REV-ERBα regulate flavivirus replication. *Nat. Commun.* **10**, 377. (doi:10.1038/s41467-019-08299-7)
9. Smith LM *et al.* 2020 An intrinsic oscillator drives the blood stage cycle of the malaria parasite *Plasmodium falciparum*. *Science* **368**, 754–759. (doi:10.1126/science.aba4357)
10. Rijo-Ferreira F, Acosta-Rodriguez VA, Abel JH, Kornblum I, Bento I, Kilaru G, Klerman EB, Mota MM, Takahashi JS. 2020 The malaria parasite has an intrinsic clock. *Science* **368**, 746–753. (doi:10.1126/science.aba2658)
11. O'Donnell AJ, Mideo N, Reece SE. 2014 Erratum to: Disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated. *Malar. J.* **13**, 503. (doi:10.1186/1475-2875-13-503)
12. O'Donnell AJ, Mideo N, Reece SE. 2013 Disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated. *Malar. J.* **12**, 372. (doi:10.1186/1475-2875-12-372)
13. O'Donnell AJ, Schneider P, McWatters HG, Reece SE. 2011 Fitness costs of disrupting circadian rhythms in malaria parasites. *Proc. R. Soc. B* **278**, 2429–2436. (doi:10.1098/rspb.2010.2457)
14. Pigeault R, Caudron Q, Nicot A, Rivero A, Gandon S. 2018 Timing malaria transmission with mosquito fluctuations. *Evol. Lett.* **2**, 378–389. (doi:10.1002/evl3.61)
15. Schneider P, Rund S.SC, Smith NL, Prior KF, O'Donnell AJ, Reece SE. 2018 Adaptive periodicity in the infectivity of malaria gametocytes to mosquitoes. *Proc. R. Soc. B* **285**, 20181876. (doi:10.1098/rspb.2018.1876)

16. Cambie G, Caillard V, Beaute-Lafitte A, Ginsburg H, Chabaud A, Landau I. 1991 Chronotherapy of malaria: identification of drug-sensitive stage of parasite and timing of drug delivery for improved therapy. *Ann. Parasitol. Hum. Comp.* **66**, 14–21. (doi:10.1051/parasite/199166114)
17. Cheng Q, Kyle DE, Gatton ML. 2012 Artemisinin resistance in *Plasmodium falciparum*: a process linked to dormancy? *Int. J. Parasitol. Drugs Drug Resist.* **2**, 249–255. (doi:10.1016/j.ijpddr.2012.01.001)
18. Hirako IC, Assis PA, Hojo-Souza NS, Reed G, Nakaya H, Golenbock DT, Coimbra RS, Gazzinelli RT. 2018 Daily rhythms of TNFα expression and food intake regulate synchrony of *Plasmodium* stages with the host circadian cycle. *Cell Host Microbe* **23**, 796–808. (doi:10.1016/j.chom.2018.04.016)
19. Prior KF *et al*. 2018 Timing of host feeding drives rhythms in parasite replication. *PLoS Pathog.* **14**, e1006900. (doi:10.1371/journal.ppat.1006900)
20. Ripperger JA, Jud C, Albrecht U. 2011 The daily rhythm of mice. *FEBS Lett.* **585**, 1384–1392. (doi:10.1016/j.febslet.2011.02.027)
21. Lamia KA, Storch KF, Weitz CJ. 2008 Physiological significance of a peripheral tissue circadian clock. *Proc. Natl Acad. Sci. USA* **105**, 15 172–15 177. (doi:10.1073/pnas.0806717105)
22. Turek FW *et al*. 2005 Obesity and metabolic syndrome in circadian clock mutant mice. *Science* **308**, 1043–1045. (doi:10.1126/science.1108750)
23. Hotta CT, Gazarini ML, Beraldo FH, Varotti FP, Lopes C, Markus RP, Pozzan T, Garcia CR. 2000 Calcium-dependent modulation by melatonin of the circadian rhythm in malarial parasites. *Nat. Cell Biol.* **2**, 466–468. (doi:10.1038/35017112)
24. Subudhi AK *et al*. 2020 Malaria parasites regulate intra-erythrocytic development duration via serpentine receptor 10 to coordinate with host rhythms. *Nat. Commun.* **11**, 2763. (doi:10.1038/s41467-020-16593-y)
25. Scheiermann C, Kunisaki Y, Frenette PS. 2013 Circadian control of the immune system. *Nat. Rev. Immunol.* **13**, 190–198. (doi:10.1038/nri3386)
26. He W *et al*. 2018 Circadian expression of migratory factors establishes lineage-specific signatures that guide the homing of leukocyte subsets to tissues. *Immunity* **49**, 1175–1190. (doi:10.1016/j.immuni.2018.10.007)
27. Allen NC, Philip NH, Hui L, Zhou X, Franklin RA, Kong Y, Medzhitov R. 2019 Desynchronization of the molecular clock contributes to the heterogeneity of the inflammatory response. *Sci. Signal* **12**, aau1851. (doi:10.1126/scisignal.aau1851)
28. Arjona A, Sarkar DK. 2005 Circadian oscillations of clock genes, cytolytic factors, and cytokines in rat NK cells. *J. Immunol.* **174**, 7618–7624. (doi:10.4049/jimmunol.174.12.7618)
29. Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. 2001 Differential functions of *mPer1*, *mPer2*, and *mPer3* in the SCN circadian clock. *Neuron* **30**, 525–536. (doi:10.1016/S0896-6273(01)00302-6)
30. Maywood E, Chesham J, Smyllie N, Hastings M. 2014 The *Tau* mutation of casein kinase 1ε sets the period of the mammalian pacemaker via regulation of Period1 or Period2 clock proteins. *J. Biol. Rhythms* **29**, 110–118. (doi:10.1177/0748730414520663)
31. Prior KF. 2018 *The evolutionary ecology of circadian rhythms in malaria parasites*. Edinburgh, UK: University of Edinburgh.
32. Prior KF, Rijo-Ferreira F, Assis PA, Hirako IC, Weaver DR, Gazzinelli RT, Reece SE. 2020 Periodic parasites and daily host rhythms. *Cell Host Microbe* **27**, 176–187. (doi:10.1016/j.chom.2020.01.005)
33. Greischar MA, Reece SE, Savill NJ, Mideo N. 2019 The challenge of quantifying synchrony in malaria parasites. *Trends Parasitol.* **35**, 341–355. (doi:10.1016/j.pt.2019.03.002)
34. Qian J, Scheer FA. 2016 Circadian system and glucose metabolism: implications for physiology and disease. *Trends Endocrinol. Metab.* **27**, 282–293. (doi:10.1016/j.tem.2016.03.005)
35. Babbitt SE, Altenhofen L, Cobbold SA, Istvan ES, Fennell C, Doerig C, Llinás M, Goldberg DE. 2012 *Plasmodium falciparum* responds to amino acid starvation by entering into a hibernatory state. *Proc. Natl Acad. Sci. USA* **109**, E3278–E3287. (doi:10.1073/pnas.1209823109)
36. Brancucci NM *et al*. 2017 Lysophosphatidylcholine regulates sexual stage differentiation in the human malaria parasite *Plasmodium falciparum*. *Cell* **171**, 1532–1544. (doi:10.1016/j.cell.2017.10.020)
37. Vollmers C, Gill S, DiTacchio L, Pulivarthy SR, Le HD, Panda S. 2009 Time of feeding and the intrinsic circadian clock drive rhythms in hepatic gene expression. *Proc. Natl Acad. Sci. USA* **106**, 21 453–21 458. (doi:10.1073/pnas.0909591106)
38. Chaix A, Lin T, Le HD, Chang MW, Panda S. 2019 Time-restricted feeding prevents obesity and metabolic syndrome in mice lacking a circadian clock. *Cell Metab.* **29**, 303–319. (doi:10.1016/j.cmet.2018.08.004)
39. Adamovich Y, Rousso-Noori L, Zwighaft Z, Neufeld-Cohen A, Golik M, Kraut-Cohen J, Wang M, Han X, Asher G. 2014 Circadian clocks and feeding time regulate the oscillations and levels of hepatic triglycerides. *Cell Metab.* **19**, 319–330. (doi:10.1016/j.cmet.2013.12.016)
40. Atger F *et al*. 2015 Circadian and feeding rhythms differentially affect rhythmic mRNA transcription and translation in mouse liver. *Proc. Natl Acad. Sci. USA* **112**, E6579–E6588. (doi:10.1073/pnas.1515308112)
41. Mistlberger RE. 1994 Circadian food-anticipatory activity: formal models and physiological mechanisms. *Neurosci. Biobehav. Rev.* **18**, 171–195. (doi:10.1016/0149-7634(94)90023-X)
42. Storch K-F, Weitz CJ. 2009 Daily rhythms of food-anticipatory behavioral activity do not require the known circadian clock. *Proc. Natl Acad. Sci. USA* **106**, 6808–6813. (doi:10.1073/pnas.0902063106)
43. Takasu NN, Kurosawa G, Tokuda IT, Mochizuki A, Todo T, Nakamura W. 2012 Circadian regulation of food-anticipatory activity in molecular clock-deficient mice. *PLoS ONE* **7**, e48892. (doi:10.1371/journal.pone.0048892)
44. Carneiro B.TS, Araujo JF. 2012 Food entrainment: major and recent findings. *Front. Behav. Neurosci.* **6**, 83.
45. Hoyle NP, O'Neill JS. 2015 Oxidation-reduction cycles of peroxiredoxin proteins and nontranscriptional aspects of timekeeping. *Biochemistry* **54**, 184–193. (doi:10.1021/bi5008386)
46. Edgar RS *et al*. 2012 Peroxiredoxins are conserved markers of circadian rhythms. *Nature* **485**, 459–464. (doi:10.1038/nature11088)
47. Feeney KA *et al*. 2016 Daily magnesium fluxes regulate cellular timekeeping and energy balance. *Nature* **532**, 375. (doi:10.1038/nature17407)
48. Rijo-Ferreira F, Pinto-Neves D, Barbosa-Morais NL, Takahashi JS, Figueiredo LM. 2017 *Trypanosoma brucei* metabolism is under circadian control. *Nat. Microbiol.* **2**, 17032. (doi:10.1038/nmicrobiol.2017.32)
49. Blanford JI, Blanford S, Crane RG, Mann ME, Paaijmans KP, Schreiber KV, Thomas MB. 2013 Implications of temperature variation for malaria parasite development across Africa. *Sci. Rep.* **3**, 1300. (doi:10.1038/srep01300)
50. Chao J, Ball GH. 1962 The effect of low temperature on *Plasmodium relictum* in *Culex tarsalis*. *J. Parasitol.* **48**, 252–254. (doi:10.2307/3275577)
51. Zheng B *et al*. 2001 Nonredundant roles of the *mPer1* and *mPer2* genes in the mammalian circadian clock. *Cell* **105**, 683-694. (doi:10.1016/S0092-8674(01)00380-4)
52. Sun Q, Zhao Y, Yang Y, Yang X, Li M, Xu X, Wen D, Wang J, Zhang J. 2017 Loss of the clock protein PER2 shortens the erythrocyte life span in mice. *J. Biol. Chem.* **292**, 12 679–12 690. (doi:10.1074/jbc.M117.783985)
53. Asher G, Sassone-Corsi P. 2015 Time for food: the intimate interplay between nutrition, metabolism, and the circadian clock. *Cell* **161**, 84–92. (doi:10.1016/j.cell.2015.03.015)
54. Mancio-Silva L *et al*. 2017 Nutrient sensing modulates malaria parasite virulence. *Nature* **547**, 213–216. (doi:10.1038/nature23009)

Paper #5 (2021a): Ecology of asynchronous asexual replication: the intraerythrocytic development cycle of *Plasmodium berghei* is resistant to host rhythms

O'Donnell and Reece *Malar J* (2021) 20:105
https://doi.org/10.1186/s12936-021-03643-z

Malaria Journal

RESEARCH

Open Access

Ecology of asynchronous asexual replication: the intraerythrocytic development cycle of *Plasmodium berghei* is resistant to host rhythms

Aidan J. O'Donnell* and Sarah E. Reece

Abstract

Background: Daily periodicity in the diverse activities of parasites occurs across a broad taxonomic range. The rhythms exhibited by parasites are thought to be adaptations that allow parasites to cope with, or exploit, the consequences of host activities that follow daily rhythms. Malaria parasites (*Plasmodium*) are well-known for their synchronized cycles of replication within host red blood cells. Whilst most species of *Plasmodium* appear sensitive to the timing of the daily rhythms of hosts, and even vectors, some species present no detectable rhythms in blood-stage replication. Why the intraerythrocytic development cycle (IDC) of, for example *Plasmodium chabaudi,* is governed by host rhythms, yet seems completely independent of host rhythms in *Plasmodium berghei,* another rodent malaria species, is mysterious.

Methods: This study reports a series of five experiments probing the relationships between the asynchronous IDC schedule of *P. berghei* and the rhythms of hosts and vectors by manipulating host time-of-day, photoperiod and feeding rhythms.

Results: The results reveal that: (i) a lack coordination between host and parasite rhythms does not impose appreciable fitness costs on *P. berghei*; (ii) the IDC schedule of *P. berghei* is impervious to host rhythms, including altered photoperiod and host-feeding-related rhythms; (iii) there is weak evidence for daily rhythms in the density and activities of transmission stages; but (iv), these rhythms have little consequence for successful transmission to mosquitoes.

Conclusions: Overall, host rhythms do not affect the performance of *P. berghei* and its asynchronous IDC is resistant to the scheduling forces that underpin synchronous replication in closely related parasites. This suggests that natural variation in the IDC schedule across species represents different parasite strategies that maximize fitness. Thus, subtle differences in the ecological interactions between parasites and their hosts/vectors may select for the evolution of very different IDC schedules.

Keywords: Periodicity, Synchrony, Circadian rhythm, Feeding timing, Intraerythrocytic development cycle, Asexual replication, Gametocyte, Transmission, Fitness

Background

Biological rhythms are a ubiquitous feature of life that enable organisms to coordinate with environmental rhythms, such as those caused by the Earth's rotation ('circadian' rhythms). Parasites from diverse taxa couple their activities to daily rhythms in the within-host

*Correspondence: aidan.odonnell@ed.ac.uk
Institute of Evolutionary Biology, and Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Charlotte Auerbach Rd, Edinburgh EH9 3FL, UK

environment, the activity patterns of hosts and vectors, as well rhythms in the abiotic environment [1, 2]. Some rhythmic activities are thought to enhance transmission, such as rhythms in the migration of filarial worm larvae (*Wuchereria bancrofti)* from the host's deep tissues to the peripheral blood capillaries at the time-of-day its vector forages for blood [3], and the coccidian parasite *Isospora turdi,* sheds at the time-of-day that minimizes mortality from UV exposure [4]. Other rhythmic activities are thought to enable parasites to cope with challenges imposed by, and exploit opportunities provided by, rhythms in the within-host environment. For example, *Botrytis cinerea,* a fungal pathogen of *Arabidopsis thaliana*, has canonical clock (transcription–translation-feedback-loop) controlled rhythms in virulence that enable it to overwhelm hosts defenses when they are upregulated in the evening [5, 6]. Similarly, *Trypanosoma brucei* uses a clock of unknown components to control gene expression of over two hundred of its genes, potentially allowing it to coordinate its metabolism with that of the host [7].

Malaria parasites are renowned for their rhythmicity, for example, the species of parasite infecting a patient can be diagnosed from the regularity of fevers [8]. Fevers are caused by the synchronous bursting (schizogony) of asexually replicating blood stage parasites when they complete an intraerythrocytic development cycle (IDC). Human malaria parasites have IDC durations of 24, 48, and 72 h and so, cause fever with matching periodicity. The rodent malaria parasite species *Plasmodium chabaudi* also exhibits a synchronous IDC (lasting 24 h) and schizogony is timed to coincide with processes related to the host's daily feeding pattern [9–11]. Specifically, IDC completion coincides with the appearance of the amino acid isoleucine in the blood (as a product of the host digesting its food), which is a nutrient the parasite must acquire from the host's food [12]. Scheduling the IDC around the availability of necessary resources is consistent with the observation that experimentally mismatching the IDC with host circadian rhythms (which dictates host activity and foraging rhythms) results in the disruption of important cellular processes, and a loss of both asexual stages and gametocytes [1, 13–15]. Furthermore, the IDC schedule determines gametocyte age at the time-of-day the mosquito vector forages for blood [16] and gametocytes exhibit time-of-day variation in their infectiousness to mosquitoes [17]. For *P. chabaudi,* it appears that by completing the IDC at night—the time-of-day that nocturnal rodents forage—asexual replication is most successful and gametocytes are also at their most infective age when vectors blood feed [17, 18].

Despite the benefits of an IDC in which parasites develop synchronously and transition between developmental stages at particular times of day, not all malaria parasite species are synchronous. Specifically, two of the four rodent malaria parasites, *Plasmodium berghei* and *Plasmodium yoelii*, have IDCs that are not a multiple of 24 h (21–23 h [19–21] and 18 h, respectively [22]), and are developmentally asynchronous, with all IDC stages occurring simultaneously in the blood throughout the host's circadian cycle (Fig. 1). Whilst the IDC schedules of *P. berghei* and *P. yoelii* are well-studied, observations suggest an asynchronous IDC occurs in some parasite species of birds [23] and lizards [24], suggesting it is a taxonomically diverse trait. Whether an infection exhibits synchronous or asynchronous replication is not dictated purely by the host because, for example, the opposing IDC schedules of *P. berghei* and *P. chabaudi* are apparent when each species infects the same age, sex, and strain of laboratory mice. This observation, coupled with recent discoveries of parasite control of the IDC schedule [25–27] suggests the degree of synchrony and the timing of the IDC are at least in part controlled by parasites. If so, it raises the possibility that a synchronous or asynchronous IDC are different parasite strategies that have evolved by natural selection because they enhance parasite fitness [18]. Synchronous or asynchronous replication is unlikely to have evolved as a consequence of abiotic environmental differences because *P. berghei* and *P. yoelii* are found in different climates (*P. berghei* in the cool African highlands, *P. yoelii* in the warmer lowlands) [28]. Nor is it likely to be imposed by the mosquito vector as both the synchronous *Plasmodium vinckei* and the asynchronous *P. berghei* have been isolated from the same species of mosquito (*Anopheles dureni millecampsi*) [28]. Notably, *P. berghei* and *P. yoelii* prefer to infect reticulocytes whereas *P. chabaudi* is a generalist and *P. vinckei* is restricted to normocytes [28]. However, it is unlikely that red blood cell age preference imposes selection for a synchronous or asynchronous IDC for several reasons. First, the human malarias *P. vivax* and to a lesser extent *P. falciparum,* prefer reticulocytes but are predominantly synchronous [29, 30]. Second, reticulocytes are released into the blood in a circadian manner [31] so a synchronous IDC intuitively appears the best way to exploit reticulocytes. Third, Deharo et al. [21] do not find that invasion of normocytes or reticulocytes affects the IDC schedule of *P. berghei*. Explaining the evolution of a synchronous or asynchronous IDC requires knowledge of whether asynchronous species benefit from this style of IDC, and so asynchrony is an adaptation (i.e. confers fitness benefits), or whether asynchronous replication is costly but these species are governed by a constraint preventing them from synchronizing their IDC.

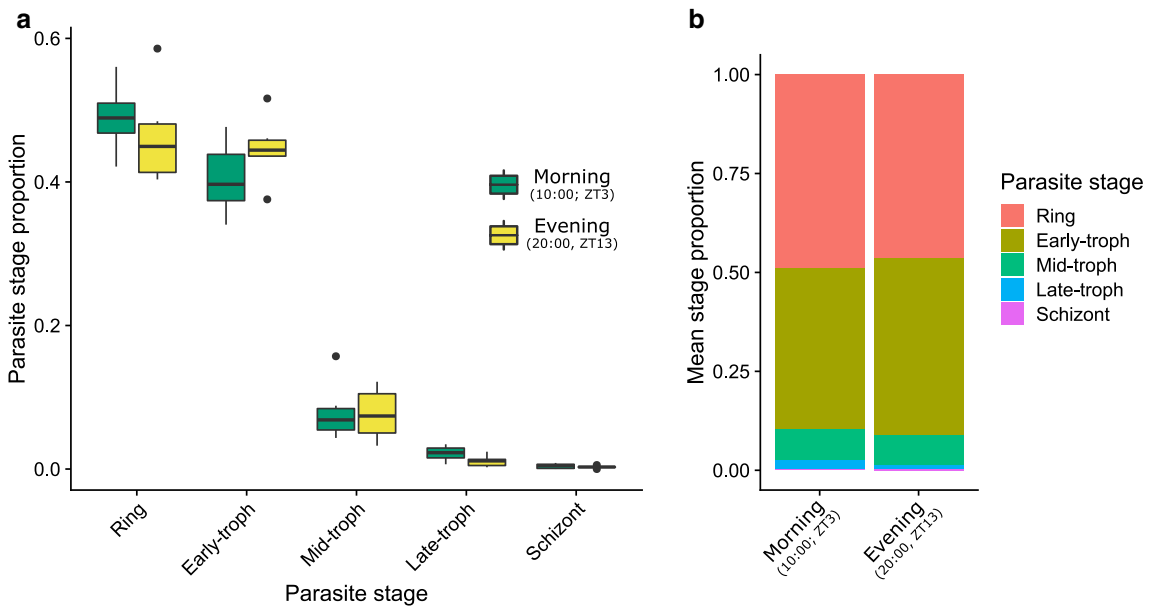

Fig. 1 *Plasmodium berghei* has an asynchronous IDC. Figure created from data in O'Donnell 2019 [49]. The proportion of parasites at each IDC stage does not differ significantly between morning (10:00 UTC; ZT3) and evening (20:00 UTC; ZT13), $\chi^2_4 = 5.83$, P = 0.21. Shown in (**a**) are median parasite stage proportions (black line) and 25–75 percentiles, with whiskers 1.5 times the interquartile range and dots representing outliers. Shown in (**b**) is the mean parasite stage proportion. Note, rings, early trophozoites (troph) and mid trophozoites compose the majority of stages observed in peripheral blood, with late trophozoites and schizonts present but in reduced numbers due to sequestration to deep tissues [3, 49, 50]. Infections (n = 6) were sampled on Day 5 PI from wild type MF1 mice in a standard 12 h light: 12 h dark photoperiod

Here *P. berghei* (strain ANKA) is used as a model for asynchronous malaria parasites. It is well known that synchronized infections of *P. berghei* can be generated via laboratory methods (e.g. inoculations of pure merozoites [21]) but synchrony is rapidly lost within a few cycles [20]. Intriguingly there are reports of synchronous *P. berghei* experimental infections in ground squirrels (with schizogony occurring at the start of the active, feeding, period which is the inverse of the schedule of *P. chabaudi*) [18, 32] and in infections of laboratory mice in which hosts were subjected to "summer-like" lighting conditions (extended light hours with wide wavelength light) [33, 34]. Mammalian host physiological responses to summer photoperiods are thought to be controlled by the pineal gland [33], a regulator of host hormones such as melatonin, which has been implicated in modulating the rate of IDC development for synchronous species [35]. Based on these observations and knowledge of the ecological factors governing the IDC schedule of *P. chabaudi*, a series of five experiments were carried out to test whether IDC of *P. berghei* can be influenced by host rhythms and if asynchronous replication has fitness consequences. The experiments asked the following questions:

1. Does the time-of-day of blood stage infection affect the densities of asexual stages and gametocytes of *P. berghei*?
2. Does the IDC of *P. berghei* become synchronized in response to changes in photoperiod?
3. Do host feeding rhythms influence the IDC schedule of *P. berghei*?
4. Are there time-of-day differences in the circulating densities of gametocytes of *P. berghei*?
5. Are there time-of-day differences in *P. berghei* infectivity to mosquitoes?

Methods

Parasites, hosts, and vectors

Hosts were either wild type (WT) MF1 mice (experiments 1, 2, 4, 5) or *Per1/2*-null clock-disrupted mice previously backcrossed onto a C57BL/6J background for over 10 generations (experiment 3), all sourced from in-house breeding colonies. PERIOD 1 (PER1) and PERIOD 2 (PER2) are essential components of the core circadian clock (i.e. the transcription–translation-feedback-loop, TTFL) and *Per1/2*-null mice are arrhythmic when placed in constant darkness [36, 37]. All experimental mice

were males, 8–10 weeks old, housed at 21 °C, and given unrestricted access to drinking water supplemented with 0.05% para-aminobenzoic acid (to supplement parasite growth, as is routine for this model system). Mice in experiments in which host light–dark and/or feeding schedules were altered (experiments 1, 2, 3) were entrained for at least 2 weeks prior to, and throughout the duration of infections. *Plasmodium berghei* (strain ANKA) was used to initiate all infections.

Parasites were administered via intraperitoneal injection (IP) at a dose of 1×10^6 parasitized red blood cells (RBC) (experiments 1–4) or 1×10^5 parasitized RBC (experiment 5). Infections were monitored by staining thin blood smears with 20% Giemsa for 20 min and asexual stages, gametocytes, IDC stage distributions (experiments 2 and 3) and gametocyte sex (experiment 4) were quantified via microscopy. Red blood cell densities were quantified using a Beckman Coulter Z2 particle counter. All infections were terminated on day 6 post infection (PI) to prevent host mortality due to complications from cerebral malaria. Parasites in all experiments were from same lineage recently transmitted through mosquitoes and cryopreserved within 1–2 passages of each other. Parasite stocks were then expanded in donor hosts before being used to initiate experimental infections. The IDC of *P. berghei* is asynchronous from frozen stocks and regardless of the number of passages between hosts.

Anopheles stephensi mosquitoes (experiment 5) were maintained under standard insectary conditions of 27 ± 1 °C, 70% humidity and 12 h light: 12 h dark photoperiod [lights on 07:00: lights off 19:00 UTC (Coordinated Universal Time)]. Female mosquitoes were randomly allocated to 2L holding cages (85 females per cage) and starved of fructose solution for 24 h before their blood meals. Each cage was exposed to an anaesthetized mouse for 30 min, all mosquitoes were able to blood feed until satiated and unfed females were removed from the cages (< 5 per cage in all cases). After feeding, mosquitoes were housed in incubators (humidity $60 \pm 5\%$) at 20.5 ± 0.5 °C and 12 h light: 12 h dark photoperiod.

Experimental designs and data collection

Experiment 1: do host rhythms have fitness consequences for P. berghei?

This experiment tested whether the IDC of *P. berghei* aligns in a cryptic way with host rhythms in a manner beneficial to it, by comparing the performance of parasites in experimental infections stemming from donor hosts with either the same phase (timing) of host and parasite rhythms, or a 12 h difference. Experimental WT mice (n = 15 per treatment) were kept in a standard (lights on 07:00: lights off 19:00 UTC) or reverse photoperiod (lights off 07:00: lights on 19:00). On day 0 PI (08:00 UTC), parasites originating from donor mice housed in the standard photoperiod were used to simultaneously infect mice in both the standard and reverse photoperiods. This created a group of infections in which inoculated parasites entered hosts at the same time (phase) in their daily rhythms as the donor host they were collected from ('matched' parasites), and another group in which parasites entered hosts at the opposite phase to their donor host ('mismatched' parasites). All mice were sampled daily, from days 2–6 post infection (PI) at 10:00 UTC to quantify asexual and sexual (gametocytes) stage densities.

Experiment 2: does the IDC become synchronous in long days?

Experiment 2 tested Arnold's [34] observation that the IDC of *P. berghei* becomes synchronous in long days. If photoperiod generates a highly synchrony IDC, at any point in the day, infections in long-day hosts (18 h light: 6 h dark) will be more synchronous (i.e. one stage dominating the stage composition) compared to infections in hosts experiencing a standard photoperiod (12 h light: 12 h dark), following [34]. Even synchronization for only part of the IDC should result in a different IDC stage distribution to parasites infecting hosts in the standard photoperiod. WT mice (n = 5 per treatment) were housed in an 18:6 schedule (lights on 16:00; lights off 10:00 UTC). On day 0 PI, infections were initiated from donor mice experiencing the same long-day photoperiod as recipient hosts. On day 6 PI (after 6–7 cycles) mice were sampled at 09:00 UTC and parasites were allocated into 5 morphologically distinct IDC stages (as per Prior et al. [11]). These data were compared to previous data from the same Zeitgeber time (ZT17) and the same UTC (09:00), but from hosts housed in a standard 12:12 photoperiod (lights on 07:00; lights off 19:00). These historical infections involved the same mouse and parasite strains, starting dose and sampling days PI, as the long-day infections. Note, the IDC of *P. chabaudi* becomes fully rescheduled to align with host rhythms after perturbations to the phase relationship between host and parasite within 7 cycles so, on top of the 5 cycles in the donor host, this design gave the IDC of *P. berghei* ample opportunity to adjust to a long-day schedule.

Experiment 3: can host feeding-associated rhythms influence the IDC schedule?

This experiment tested whether the IDC schedule of *P. berghei* can be perturbed by altering the time-of-day that hosts feed [9–11]. TTFL-clock disrupted mice housed in constant darkness were used as hosts to ensure that parasites only experienced rhythms stemming from host feeding-related rhythms. If host feeding-related rhythms

influence *P. berghei* in the same manner as *P. chabaudi,* the IDC of *P. berghei* will become synchronous and schizogony will occur during the second half of the window in which hosts feed. *Per1/2*-null (n = 4 per treatment) mice were housed in DD (continuous darkness) with constant dim red LED light. Hosts had access to food constantly (all-day fed) or only during a window of 8 h per day (food in 09:00/food out 17:00 UTC; time restricted fed; TRF). Note, TRF protocols do not lead to caloric restriction or a loss in body mass [10]. Food was provided/removed by changing the cage lid and sweeping the cage for stray pellets at the times of removal and mice in the all-day fed group experienced the same disturbance. On days 5–6 PI, parasites were sampled from 12:00 UTC every 4 h for 28 h. Both the number of parasites at ring-trophozoite stage ("rings") and the total observed were recorded.

Experiment 4: do transmission traits show time-of-day variation?

This experiment probed for rhythmicity in reproductive traits that underpin transmission, specifically gametocyte density, exflagellation rate and ookinete density. WT mice (n = 5) experienced a standard photoperiod (lights on 07:00; lights off 19:00 UTC). On day-2 PI, mice were treated with a 30 mg/kg dose of phenylhydrazine hydrochloride (PHZ) via IP injection to induce reticulocytosis and promote gametocyte conversion [38]. On days 5 and 6 post infection, parasites were sampled from each infection at 08:00 UTC (ZT1) and then every 4 h for 24 h. At each sampling point, 2 µl blood samples were diluted in 100 µl ookinete culture media (RPMI-1640 medium containing 10% fetal calf serum, pH 8). After 10 min, a 0.3 µl sub-sample was observed on a haemocytometer and the number of exflagellation events counted over 10 min. At each sampling point, gametocytes were sexed (determined by colour and morphology) and their densities quantified via thin blood smear. Finally, at each sampling point, a second 2 µl blood sample was diluted in 200 µl ookinete culture media, incubated for 24 h at 19 °C and the number of ookinetes in a 0.3 µl sub-sample was counted using a haemocytometer. Exflagellation events and ookinete counts were normalized between samples (exflagellations per male and ookinetes per female) by dividing the counts by the number of male/female gametocytes in the 0.3 µl culture sample they were derived from (gametocytaemia × (RBC density per ml × sample volume)).

Experiment 5: are oocyst densities influenced by the time-of-day of transmission?

Plasmodium chabaudi demonstrates time-of-day variation in infectivity, likely as a consequence of the IDC schedule dictating the age range of gametocytes at the time of transmission [17]. This experiment tested whether *P. berghei* also displays time-of-day variation in infectivity to mosquitoes. If rhythmicity in transmission traits exists and is adaptive (i.e. benefits fitness), parasites transmitted at night are predicted to be more successful. Mosquito cages (6 cages per treatment), each housing 85 female mosquitoes, were randomly allocated to receive blood meals from infected mice (n = 6 per treatment) experiencing their morning (10:00 UTC; ZT3) or evening (20:00 UTC; ZT13) on day 6 PI. This created two groups of infections that varied by time-of-day, for all parties. On day 14 post blood meal, 15 mosquitoes per cage were assessed for oocyst prevalence. Specifically, midguts were dissected, stained for 2 min in 0.5% mercurochrome, washed in PBS and the number of oocysts per midgut counted via microscopy. Circulating gametocyte densities were determined by thin blood smear just prior to mosquitoes feeding on each host.

Data analysis

The effects of time-of-day, light:dark photoperiod, and host feeding regime on parasite densities and IDC stage proportions were compared between groups using linear mixed-effect models with mouse identity fitted as a random effect. Parasite densities in experiment 1 and gametocyte densities in experiment 5 were analysed using linear models. Oocyst densities in experiment 5 were square root transformed to meet assumptions of normality and homogeneity of variance. Models were selected using step-wise selection via the drop1 function in R. Whether the dynamics of transmission stage metrics in experiment 4 are consistent with ~ 24-h rhythms was assessed using a harmonic regression approach via Circwave (v. 1.4, courtesy of R. Hut; http://www.euclock.org) and confirmed using an alternative non-parametric algorithm via JTK_CYCLE [39]. All other statistical analyses were carried out using R version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Experiment 1: do host rhythms have fitness consequences for P. berghei?

Overall performance, as measured by cumulative asexual density, did not differ significantly between parasites stemming from donor hosts 'matched' or 'mismatched' to the timing of rhythms in recipient hosts (Fig. 2a; $F_{(1,28)} = 0.10$, $P = 0.75$; mean cumulative asexual density per ml blood $\times 10^8 \pm SEM = 7.04 \pm 0.25$). Similarly, cumulative gametocyte density/ml did not differ significantly between 'matched' and 'mismatched' infections (Fig. 2b; $F_{(1,28)} = 3.70$, $P = 0.06$; mean cumulative gametocyte density per ml blood $\times 10^7 \pm SEM = 1.17 \pm 0.09$). In the infection dynamics, there was a significant interaction

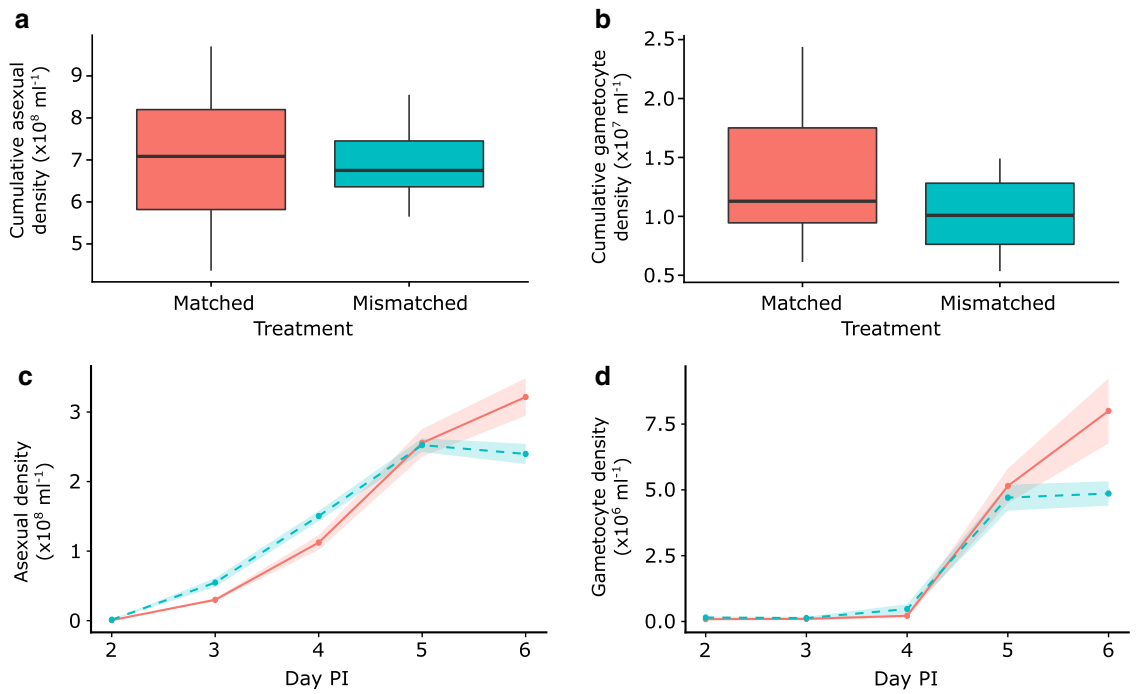

Fig. 2 Cumulative (top) and daily (bottom) dynamics for the densities of asexual (**a**, **c**) and sexual stages per ml blood (**b**, **d**). Infections (n = 15) were initiated in wild type MF1 mice with parasites from donor hosts whose rhythms were either 'matched' (morning donor/morning recipient) or 'mismatched' (morning donor/evening recipient") to the rhythms of recipient hosts. Shown are (a,b) median parasite densities (black line) and 25–75 percentiles, with whiskers 1.5 times the interquartile range and dots representing outliers and (**c**, **d**) mean with SEM in shading

between treatment and time, in which asexual stages (Fig. 2c; treatment:day: $\chi^2_4 = 28.57$, $P < 0.001$) and gametocytes (treatment:day: $\chi^2_4 = 17.38$, $P = 0.002$; Fig. 2d) varied over time. This is driven solely by the divergence of treatment groups on day 6, in which asexual densities and gametocytes are on average 25% and 40%, respectively, lower in host-mismatched infections (models without day 6 PI; asexual treatment:day: $\chi^2_3 = 7.72$, $P = 0.052$, gametocyte treatment:day: $\chi^2_3 = 1.55$, $P = 0.67$).

Experiment 2: does the IDC become synchronous in long days?

The IDC of parasites in hosts housed in a long-day 18:6 light:dark photoperiod did not become synchronized. Specifically, IDC stage proportions in long-day infections followed the distribution observed previously in standard 12:12 photoperiod infections, which does not differ significantly between morning (ZT2) and evening (ZT17) (Fig. 3, interaction between photoperiod and parasite stage: $\chi^2_8 = 5.22$, $P = 0.73$). Parasite stage composition was primarily made up of rings, early trophozoites (trophs) and mid-trophs (mean stage proportion ± SEM: rings = 0.34 ± 0.02, early-trophs = 0.34 ± 0.02, mid-trophs = 0.27 ± 0.02) with late-trophs and schizonts likely sequestering (late-trophs = 0.05 ± 0.01, schizonts = 0.01 ± 0.002).

Experiment 3: can host feeding-associated rhythms influence the IDC schedule?

The IDC did not become synchronized or display altered timing in hosts with strong feeding rhythms (TRF) compared to all-day fed hosts (Fig. 4a). Specifically, the proportion of parasites at ring stage was not significantly affected by time-of-day ($\chi^2_1 = 0.31$, $P = 0.58$), host-feeding schedule ($\chi^2_1 = 0.92$, $P = 0.34$), or their interaction ($\chi^2_1 = 1.37$, $P = 0.24$). The proportion of ring stages remained fairly constant through the 28-h sampling window at 33.5% (±0.01 SEM). The IDC schedule can also be assessed via the density of developmental stages [40]. Ring stage densities did not differ significantly between all-day fed and TRF mice (host feeding schedule:time interaction: $\chi^2_1 = 2.91$, $P = 0.09$ and main effect $\chi^2_1 = 1.27$, $P = 0.26$), but ring stage densities did increase over time ($\chi^2_1 = 4.66$, $P = 0.03$). This is simply due to replication causing parasite density to increase (specifically, by 80.1% (±26.8 SEM)) as infections aged during the sampling time series. Thus, cumulative densities varied over the 28 h sampling window (time: $\chi^2_1 = 178.08$, $P < 0.001$,

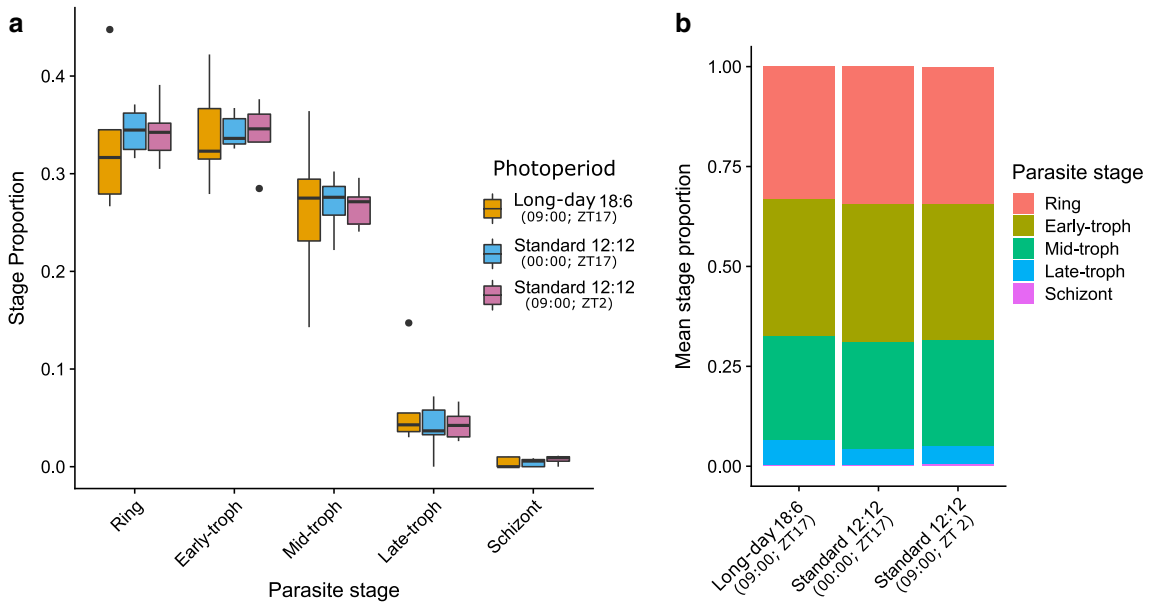

Fig. 3 IDC stage distributions for parasites in hosts subjected to a long-day 18:6 light:dark photoperiod vs a standard 12:12 photoperiod. Infections were compared to the stage distributions of infections subjected to a standard 12 h light: 12 h dark photoperiod when sampled at midnight (which is the same ZT as the long-day infections were sampled), and at 09:00 UTC (which is the same UTC as the long-day infections were sampled). **a** Median parasite stage proportions (black line) and 25–75 percentiles, with whiskers 1.5 times the interquartile range and dots representing outliers, and **b** parasite stage distribution illustrated as mean stage proportions. Infections (n = 6) were sampled on days 5–6 PI from wild type MF1 mice

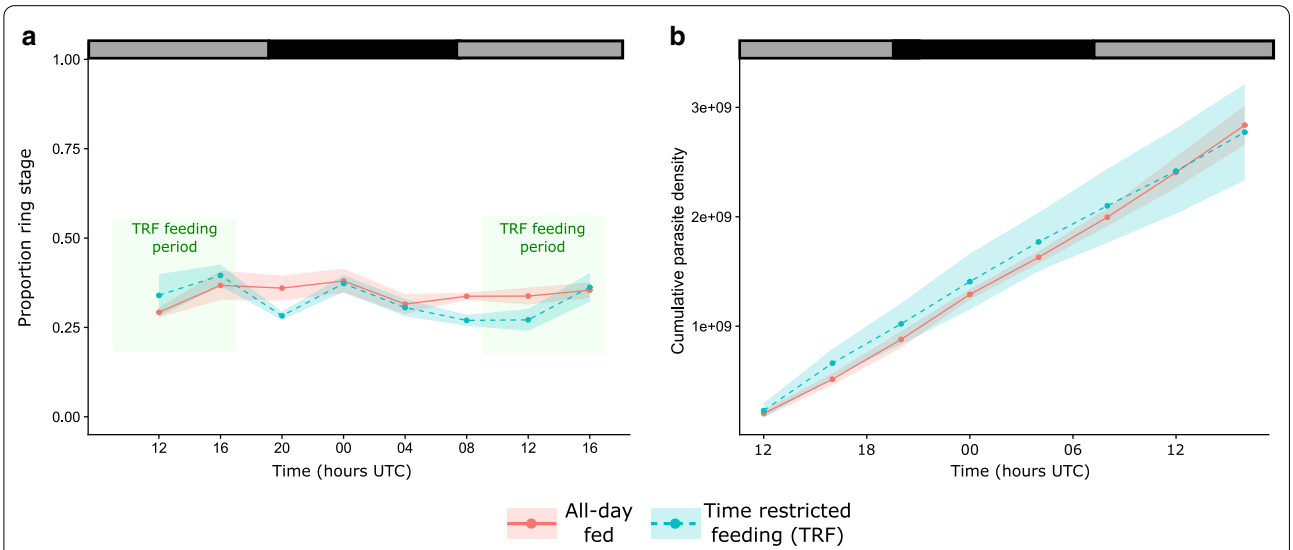

Fig. 4 IDC rhythms and cumulative parasite densities in hosts with (TRF) and without (all-day fed) feeding and food-associated rhythms. All hosts were arrhythmic Per1/2-null mice (n = 4), either given access to food continuously (all-day fed; solid line) or during an 8 h window each day (time restricted feeding (TRF); dashed line). Shown are (**a**) the mean ring stage proportion ± SEM in shading and (**b**) mean cumulative parasite density ± SEM in shading (0-28 h sampling period; days 5-6PI). Green boxes in **a** represent period when food is available to TRF mice. All mice were housed in continuous darkness so black and grey bars represent day and night UTC

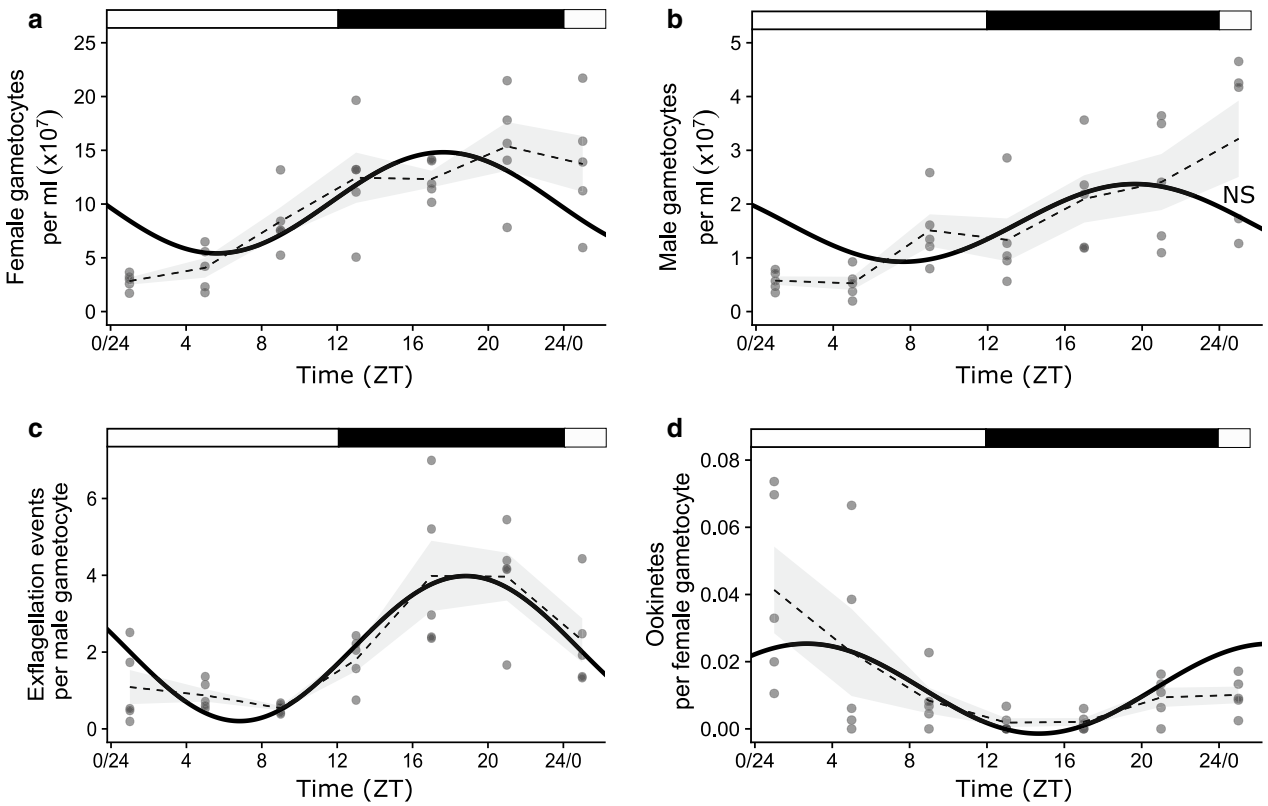

Fig. 5 Time-of-day variation in *P. berghei* transmission traits. **a** Female gametocyte densities, **b** male gametocyte densities, **c** exflagellation events per male gametocyte and **d** number of ookinetes per female gametocyte. Shown are fitted 24 h Circwave rhythms (solid lines) with mean ± SEM (dashed line and shading) and individual infection data (points). Fits that are non-significant (P > 0.05) are labelled NS and provided for illustration of the trend. Black and white bars represent lights ON (white) and lights OFF (black) and time is given in Zeitgeber time (ZT) in which ZT0 = lights on. Infections (n = 5) were sampled every 4 h on days 5-6PI from wild type MF1 mice pre-treated with phenylhydrazine

treatment: $\chi^2_1 = 0.05$, P = 0.83), but not in a manner that differed significantly between the TRF and all-day fed hosts (Fig. 4b, interaction $\chi^2_1 = 1.26$, P = 0.26).

Experiment 4: do transmission traits show time-of-day variation?

Both the densities of male ($\chi^2_1 = 34.53$, P < 0.001) and female ($\chi^2_1 = 36.63$, P < 0.001) gametocytes varied during the 24 h sampling window (Fig. 5a, b). For female gametocytes, the pattern is consistent with a 24 h rhythm (Circwave: $F_{(2,32)} = 7.01$, P = 0.003, JTK_Cycle: BH.Q = 0.04, P = 0.03), with peak density occurring in the evening (ZT17) and a peak-to-trough amplitude of 9.4×10^7 gametocytes per ml. The pattern for males is visually similar but does not fit a 24 h rhythm (Circwave: $F_{(2,32)} = 2.77$, P = 0.08, JTK_Cycle: BH.Q = 0.20, P = 0.20). In contrast, the number of exflagellation events per male varied during the sampling window (Fig. 5c; $\chi^2_1 = 13.57$, P < 0.001) and fitted a 24 h rhythm (Circwave: $F_{(2,32)} = 19.30$, P < 0.001, JTK_Cycle: BH.Q < 0.001, P < 0.001), with ~ 4 × more exflagellation (peak-to-trough amplitude = 3.78 exflagellation events) in the evening (ZT20) than during the day. Similarly, the number of ookinetes per female varied during the sampling window (Fig. 5d; $\chi^2_1 = 8.56$, P = 0.003), fitting a 24 h rhythm (Circwave: $F_{(2,32)} = 5.35$, P = 0.01,, JTK_Cycle: BH.Q = 0.003, P = 0.002). Peak density of ookinetes occurred in the morning (ZT 4) rather than the late evening and the rhythm exhibited a peak-to-trough amplitude of 0.03 ookinetes per female.

Experiment 5: are oocyst densities influenced by the time-of-day of transmission?

First, there were no significant differences in the densities gametocytes on day 6 PI between infections that were transmitted in the morning (10:00 UTC; ZT3) or the evening (20:00 UTC; ZT13; $F_{(1,10)} = 0.17$, P = 0.69). Note, hosts in this experiment did not receive PHZ and so, gametocyte densities were lower than in experiment 4. Second, oocyst burden did not differ significantly

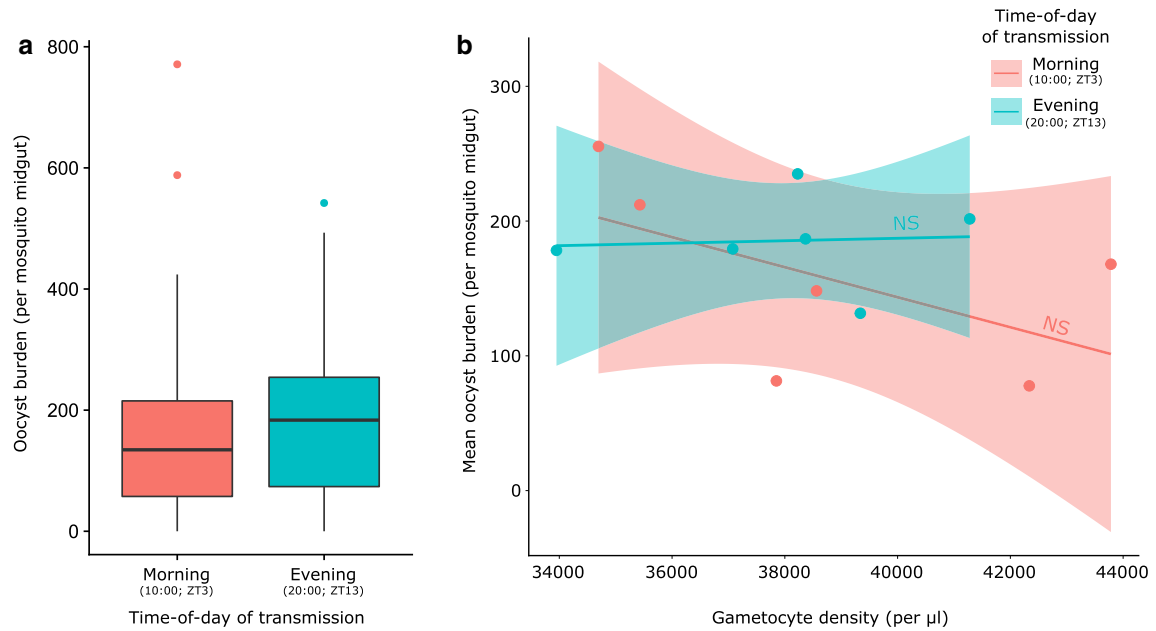

Fig. 6 Oocyst burden and gametocyte infectiousness do not vary between morning and evening. Mosquito blood meals occurred when both infected mice and mosquitoes were experiencing their morning (10:00 UTC; ZT3) or their evening (20:00 UTC; ZT13). Shown are (**a**) median oocyst burdens (black line; n = 90) and 25–75 percentiles, with whiskers 1.5 times the interquartile range and dots representing outliers or (**b**) mean oocyst burdens (points; individuals grouped by time-of-day of transmission) with lines indicating non-significant (NS) linear regressions for illustration of trends (SEM in shading; n = 6)

between morning and evening transmissions, with an overall mean oocyst burden of 171.3 (±9.84 SEM) oocysts per midgut (Fig. 6a, $\chi_1^2 = 0.59$, P = 0.44). Furthermore, variation in oocyst burden could not be attributed to variation in gametocyte density (Fig. 6b, $\chi_1^2 = 2.95$, P = 0.09) or its interaction with the time-of-day of transmission ($\chi_1^2 = 0.77$, P = 0.38), suggesting that the infectiousness of gametocytes does not vary across the day.

Discussion

The experiments presented here probe, in several ways, whether the IDC of the asynchronous parasite species *P. berghei,* can be synchronized by perturbations to host rhythms, and whether there are fitness consequences of asynchronous replication. The results reveal that the IDC of *P. berghei* is resistant to being synchronized or scheduled by either long photoperiod days (Fig. 3) or by host feeding-related rhythms (Fig. 4). Furthermore, there is little evidence that host time-of-day affects the within-host component of *P. berghei* fitness. Specifically, the performance of infections (i.e. cumulative densities of asexual stages and gametocytes) is not significantly affected by a "phase-shift" (mismatch) from donor to recipient hosts (Fig. 2). Whilst densities of both asexuals and gametocytes are lower in mismatched infections on day 6 this is unlikely to represent a substantial fitness effect related to host rhythms. First, this drop is not sufficient to affect the cumulative counts. Second, *P. chabaudi* displays a much greater cost of mismatch which is caused by events in the first two cycles that become exacerbated as infections pass through successive cycles of replication [13, 14]. Thus, near identical trajectories for asexual and gametocyte densities until day 6 PI for *P. berghei* is not consistent with a prolonged impact of host rhythms. It remains possible that host rhythms impact *P. berghei* and *P. chabaudi* differently, but this requires the host rhythm in question either to be absent from *P. chabaudi* infections or occur 4–5 days sooner than in *P. berghei* infections.

The consequences of time-of-day for the between-host (i.e. transmission) component of *P. berghei* fitness is more complicated. Whilst the densities of female gametocytes and the ability of males to exflagellate vary throughout the day with similar patterns, temporal variation in ookinete production follows a damper rhythm with a different pattern (Fig. 5). This suggests that any rhythmicity in the activities of sexual stages is eroded by the time parasites have developed to ookinetes—the ookinete rhythm observed opposes that for males and females and is small, with ookinete prevalence varying only by ~ 2–3% throughout the day. It is unclear what drives the time-of-day variation in the densities of females and exflagellation rates of males. If gametocytes are produced from

merozoites stemming from schizogony events occurring at all times-of-day, and gametocytes follow the same developmental rates, then there should be little periodicity in their number or abilities. However, host immune rhythms may influence gametocyte mortality/fertility, imposing rhythms on intrinsically arrhythmic gametocytes. Or perhaps *P. berghei* gametocytes are able to modulate their developmental rate to synchronize maturity. The effects of time-of-day on the densities of females and exflagellation rates of males are small, so may not be biologically relevant, especially by the oocyst stage of transmission, because the time-of-day of transmission does not influence gametocyte infectivity or oocyst density (Fig. 6).

That *P. berghei* fitness is not affected by a "phase-shift" (mismatch) between donor and recipient hosts provides a clue to the costs of mismatch for *P. chabaudi* infections initiated with ring stages. For example, *P. chabaudi* infections initiated with mismatched ring stages could perform poorly because hosts mount better defences against evening invaders. If this were the case, the phenomenon should apply to *P. berghei* too, but it does not. This suggests that mis-timing of the IDC itself (e.g. being out of synch with resources needed for development) is costly to *P. chabaudi* from the outset of infection [18]. However, the results do not shed light on why the IDC of *P. berghei* is resistant to host time-of-day. There are many possible explanations for why a life history trait differs across species. In this case, the explanation depends on whether the asynchronous IDC of *P. berghei* is an adaption (i.e. enhances fitness), is selectively neutral (little effect on fitness), or is a constraint (deleterious but unavoidable). To explore the evolutionary context, it is helpful to consider the IDC as a series of three traits—its level of synchrony, timing of transitions between IDC stages, and the duration of the IDC.

First, how might an asynchronous IDC be an adaption? Faster replication (which enhances competitive ability and within-host survival) is possible from an IDC with a short duration, compared to having an IDC constrained to 24 h (or multiples of) by the need to coordinate with host rhythms. A short IDC is by definition unable to coordinate with 24 h environmental periodicity. Perhaps the benefits of fast replication outweigh the costs of not coordinating with host rhythms, or somehow species with a short IDC are not affected by host rhythms. Perhaps *P. berghei* is able to acquire and store resources through the IDC and so, is not reliant on certain nutrients appearing in the blood when it gets to a certain IDC stage(s)? If so, the question becomes why is *P. chabaudi* unable to achieve this too? An answer might lie in the different within host densities these species reach. Many *P. chabaudi* strains can reach 30–80% peak parasitaemia as late as day 10 PI (depending on starting dose) without host mortality, whereas *P. berghei* tends to kill the host on days 6–8 PI (irrespective of starting dose) due to cerebral malaria, having only reached parasitaemias far lower than 30%. Thus, *P. chabaudi* may require a lot of resources from the host to reach this high biomass, creating a need to efficiently exploit host rhythms, but *P. berghei*'s resource needs might be low enough to be met at any time-of-day. How likely this scenario is, depends on the extent to which development is limited by the resources available within individual RBC versus the blood environment as a whole. For instance, the much greater production of merozoites per schizont by *P. berghei* (6–8 for *P. chabaudi* and 12–18 for *P. berghei*) would intuitively suggest *P. berghei* has greater resource needs from each RBC.

Second, extreme synchrony and extreme asynchrony might be equally good ("alternative") strategies in a rhythmic environment, with intermediate levels of synchrony being selected against [41]. Synchrony may bring benefits of coordination with host feeding rhythms but be costly in terms of coinciding with rhythmic immune responses that have IDC-stage-specific effects. For example, in human malaria infections, γδ T cells exhibit daily rhythms [42, 43] and effectively target *P. falciparum* merozoites [44]. Asynchrony might protect parasites against immune rhythms but come at the cost of loss of coordination with host feeding rhythms. An asynchronous IDC could also be selectively neutral if *P. berghei* has different resource requirements to *P. chabaudi*, in that the nutrients *P. berghei* needs are not limiting at any time-of-day. Recent work suggests the IDC schedule of *P. chabaudi* is specifically tied to rhythms in blood isoleucine concentration resulting from the host digesting its food [12]. However, amino acid usage patterns in *P. berghei* and *P. chabaudi* are very similar [45], suggesting that if an isoleucine rhythm favours a synchronous and timed IDC in *P. chabaudi*, this should also be the case for *P. berghei*. Perhaps residing in reticulocytes dampens rhythmicity in the resources *P. berghei* needs? Whilst any differences in the ecology of rhythms between *P. berghei* and *P. chabaudi* infections remain unknown, if there are no benefits from a synchronous and timed IDC, natural variation in IDC duration between individual parasites will quickly erode an IDC schedule, perhaps explaining why synchrony is rapidly lost in *P. berghei* infections initiated with a single IDC stage.

Third, *P. berghei* might be under some constraints in murine hosts where it is unable to control its IDC schedule to its detriment. For example, the amplitude of daily rhythms in isoleucine in well fed lab mice may not be sufficient to allow *P. berghei* to tell the time (if *P. chabaudi* is more sensitive to this time cue). This scenario could be tested in 2 ways. First, by probing if the withdrawal of isoleucine from culture media stalls IDC completion of *P.*

berghei as it does for *P. chabaudi* and *P. falciparum* [12, 46]. Second, by comparing the performance of asynchronous and artificially synchronized *P. berghei* infections. This is more challenging than it intuitively seems because fitness needs to be assessed within the first few cycles from low density infections before synchrony degrades, and also, the confounding handling effects involved in preparing each type of infection are hard to control for. If asynchronous and synchronous infections can be fairly compared, synchronized infections will perform better if the IDC of *P. berghei* is constrained to be asynchronous.

Conclusion

The experiments presented here were designed to assess whether host/vector rhythms matter to the IDC of *P. berghei*, rather than explain the ecology underpinning an asynchronous IDC, for which more work is required. This study demonstrates that the IDC of *P. berghei* is resistant to being synchronized and scheduled by environmental photoperiod and by host feeding-related rhythms, and that time-of day has very minor, if any, effects on its fitness. This finding supports recent studies suggesting that across *Plasmodium spp.* features of the IDC schedule are under the control of parasite genes [25–27], rather than directly generated by the host, by for example selectively removing certain IDC stages at certain times of day. Why some species are impervious to the daily rhythms of their hosts and vectors remains mysterious. Further work might benefit from confirming the IDC schedule of *P. berghei* is also asynchronous in the natural rodent host *Grammomys surdaster* (infection dynamics in these rats do those of mirror lab mice [47]) or even bats as *P. berghei* may have a stronger coevolutionary relationship with bats than rodents [48]. Another approach could involve testing whether, unlike *P. chabaudi*, *P. berghei* has adapted to store resources that are rhythmically provided by the host, thus facilitating IDC completion at any time-of-day. Understanding the costs and benefits of different IDC schedules is central to the success of any interventions that intentionally, or unintentionally, disrupt the timing, synchrony, and duration of the IDC.

Acknowledgements
We thank Ronnie Mooney, Kim Prior, and Aidan McHall-Hagan for technical assistance, and Petra Schneider, Jacob Holland and Giles K. P. Bara for advice.

Author's contributions
AOD and SR conceived and designed the project. AOD carried out the experiments. All authors interpreted the data, prepared the manuscript and approved the final manuscript. All authors read and approved the final manuscript.

Funding
This work was supported by the Wellcome Trust (202769/Z/16/Z; 204511/Z/16/Z), the Royal Society (UF110155; NF140517), and the Human Frontier Science Program (RGP0046/2013).

Availability of data and materials
The datasets supporting the conclusions of this article are available in the Edinburgh DataShare repository: https://datashare.ed.ac.uk/handle/10283/3204

Ethics approval and consent to participate
All procedures were carried out in accordance with the UK Home Office regulations (Animals Scientific Procedures Act 1986; project license number: 70/8546) and approved by the University of Edinburgh.

Competing interests
None declared.

Received: 16 September 2020 Accepted: 10 February 2021
Published online: 19 February 2021

References

1. Reece SE, Prior KF, Mideo N. The life and times of parasites: rhythms in strategies for within-host survival and between-host transmission. J Biol Rhythms. 2017;32:516–33.
2. Westwood ML, O'Donnell AJ, de Bekker C, Lively CM, Zuk M, Reece SE. The evolutionary ecology of circadian rhythms in infection. Nat Ecol Evol. 2019;3:552–60.
3. Hawking F. The 24-hour periodicity of microfilariae: biological mechanisms responsible for its production and control. Proc Sci Biol. 1967;169:59–76.
4. Martinaud G, Billaudelle M, Moreau J. Circadian variation in shedding of the oocysts of *Isospora turdi* (Apicomplexa) in blackbirds (*Turdus merula*): an adaptative trait against desiccation and ultraviolet radiation. Int J Parasitol. 2009;39:735–9.
5. Hevia MA, Canessa P, Müller-Esparza H, Larrondo LF. A circadian oscillator in the fungus *Botrytis cinerea* regulates virulence when infecting *Arabidopsis thaliana*. Proc Natl Acad Sci USA. 2015;112:8744–89.
6. Larrondo LF, Canessa P. The clock keeps on ticking: emerging roles for circadian regulation in the control of fungal physiology and pathogenesis. Curr Top Microbiol Immunol. 2019;422:121–56.
7. Rijo-Ferreira F, Pinto-Neves D, Barbosa-Morais NL, Takahashi JS, Figueiredo LM. *Trypanosoma brucei* metabolism is under circadian control. Nat Microbiol. 2017;2:17032.
8. Garcia CR, Markus RP, Madeira L. Tertian and quartan fevers: temporal regulation in malarial infection. J Biol Rhythms. 2001;16:436–43.
9. Hirako IC, Assis PA, Hojo-Souza NS, Reed G, Nakaya H, Golenbock DT, et al. Daily rhythms of TNFalpha expression and food intake regulate synchrony of *Plasmodium* stages with the host circadian cycle. Cell Host Microbe. 2018;23(796–808):e6.
10. O'Donnell AJ, Prior KF, Reece SE. Host circadian clocks do not set the schedule for the within-host replication of malaria parasites. Proc Soc Biol. 2020;287:20200347.
11. Prior KF, van der Veen DR, O'Donnell AJ, Cumnock K, Schneider D, Pain A, et al. Timing of host feeding drives rhythms in parasite replication. PLoS Pathog. 2018;14:e1006900.
12. Prior KF, Middleton B, Owolabi AT, Westwood ML, Holland J, O'Donnell AJ, et al. An essential amino acid synchronises malaria parasite development with daily host rhythms. bioRxiv 2020:2020.2008.2024.264689.
13. O'Donnell AJ, Mideo N, Reece SE. Erratum to: disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated. Malar J. 2014;13:503.
14. O'Donnell AJ, Mideo N, Reece SE. Disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated. Malar J. 2013;12:372.
15. O'Donnell AJ, Schneider P, McWatters HG, Reece SE. Fitness costs of disrupting circadian rhythms in malaria parasites. Proc Biol Sci. 2011;278:2429–36.
16. Westwood ML, O'Donnell AJ, Schneider P, Albery GF, Prior KF, Reece SE. Testing possible causes of gametocyte reduction in temporally out-of-synch malaria infections. Malar J. 2020;19:17.
17. Schneider P, Rund SSC, Smith NL, Prior KF, O'Donnell AJ, Reece SE. Adaptive periodicity in the infectivity of malaria gametocytes to mosquitoes. Proc Soc Biol. 2018;285:20181876.

18. Prior KF, Rijo-Ferreira F, Assis PA, Hirako IC, Weaver DR, Gazzinelli RT, et al. Periodic parasites and daily host rhythms. Cell Host Microbe. 2020;27:176–87.
19. Janse CJ, Waters AP. *Plasmodium berghei* - The application of cultivation and purification techniques to molecular studies of malaria parasites. Parasitol Today. 1995;11:138–43.
20. Mons B, Janse CJ, Boorsma EG, Vanderkaay HJ. Synchronized erythrocytic schizogony and gametocytogenesis of *Plasmodium berghei* in vivo and in vitro. Parasitology. 1985;91:423–30.
21. Deharo E, Coquelin F, Chabaud AG, Landau I. The erythrocytic schizogony of two synchronized strains of *Plasmodium berghei*, NK65 and ANKA, in normocytes and reticulocytes. Parasitol Res. 1996;82:178–82.
22. Gautret P, Deharo E, Tahar R, Chabaud AG, Landau I. The adjustment of the schizogonic cycle of *Plasmodium chabaudi chabaudi* in the blood to the circadian rhythm of the host. Parasite. 1995;2:69–74.
23. Valkiūnas G, Iezhova TA. Keys to the avian malaria parasites. Malar J. 2018;17:212.
24. Thompson PE, Huff CG. Saurian malarial parasites of the United States and Mexico. J Infect Dis. 1944;74:68–79.
25. Rijo-Ferreira F, Acosta-Rodriguez VA, Abel JH, Kornblum I, Bento I, Kilaru G, et al. The malaria parasite has an intrinsic clock. Science. 2020;368:746–53.
26. Smith LM, Motta FC, Chopra G, Moch JK, Nerem RR, Cummins B, et al. An intrinsic oscillator drives the blood stage cycle of the malaria parasite *Plasmodium falciparum*. Science. 2020;368:754–9.
27. Subudhi AK, O'Donnell AJ, Ramaprasad A, Abkallo HM, Kaushik A, Ansari HR, et al. Malaria parasites regulate intra-erythrocytic development duration via serpentine receptor 10 to coordinate with host rhythms. Nat Commun. 2020;11:2763.
28. Killick-Kendrick R, Peters W. Rodent malaria. London: Academic Press; 1978.
29. Kitchen S. The infection of reticulocytes by *Plasmodium vivax*. Am J Trop Med Hyg. 1938;18:347–59.
30. Pasvol G, Weatherall D, Wilson R. The increased susceptibility of young red cells to invasion by the malarial parasite *Plasmodium falciparum*. Br J Haematol. 1980;45:285–95.
31. Savill NJ, Chadwick W, Reece SE. Quantitative analysis of mechanisms that govern red blood cell age structure and dynamics during anaemia. PLoS Comput Biol. 2009;5:e1000416.
32. Ramakrishnan S, Prakash S. Susceptibility of the Indian garden squirrel (*Sciurus palmarum*) to *Plasmodium berghei* and its asexual periodicity. Nature. 1951;167:533.
33. Arnold JD, Berger A, Martin DC. The role of the pineal in mediating photoperiodic control of growth and division synchrony and capillary sequestration of *Plasmodium berghei* in mice. J Parasitol. 1969;55:609–16.
34. Arnold JD, Lalli F, Martin DC. Augmentation of growth and division synchrony and of vascular sequestration of *Plasmodium berghei* by the photoperiodic rhythm. J Parasitol. 1969;55:597–608.
35. Hotta CT, Gazarini ML, Beraldo FH, Varotti FP, Lopes C, Markus RP, et al. Calcium-dependent modulation by melatonin of the circadian rhythm in malarial parasites. Nat Cell Biol. 2000;2:466–8.
36. Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron. 2001;30:525–36.
37. Maywood E, Chesham J, Smyllie N, Hastings M. The Tau mutation of casein kinase 1ϵ sets the period of the mammalian pacemaker via regulation of Period1 or Period2 clock proteins. J Biol Rhythms. 2014;29:110–8.
38. Gautret P, Coquelin F, Chabaud AG, Landau I. The production of gametocytes by rodent *Plasmodium* species in mice during phenylhydrazine induced reticulocytosis. Acta Parasitol. 1997;42:65–7.
39. Hughes ME, Hogenesch JB, Kornacker K. JTK_CYCLE: an efficient nonparametric algorithm for detecting rhythmic components in genome-scale data sets. J Biol Rhythms. 2010;25:372–80.
40. Greischar MA, Reece SE, Savill NJ, Mideo N. The challenge of quantifying synchrony in malaria parasites. Trends Parasitol. 2019.
41. Greischar MA, Read AF, Bjornstad ON. Synchrony in malaria infections: how intensifying within-host competition can be adaptive. Am Nat. 2014;183:E36-49.
42. Carding SR, Egan PJ. γδ T cells: functional plasticity and heterogeneity. Nat Rev Immunol. 2002;2:336–45.
43. Scheiermann C, Gibbs J, Ince L, Loudon A. Clocking in to immunity. Nat Rev Immunol. 2018;18:423–37.
44. Costa G, Loizon S, Guenot M, Mocan I, Halary F, de Saint-Basile G, et al. Control of *Plasmodium falciparum* erythrocytic cycle: γδ T cells target the red blood cell–invasive merozoites. Blood. 2011;118:6952–62.
45. Yadav MK, Swati D. Comparative genome analysis of six malarial parasites using codon usage bias based tools. Bioinformation. 2012;8:1230.
46. Babbitt SE, Altenhofen L, Cobbold SA, Istvan ES, Fennell C, Doerig C, et al. *Plasmodium falciparum* responds to amino acid starvation by entering into a hibernatory state. Proc Natl Acad Sci USA. 2012;109:E3278–87.
47. Conteh S, Anderson C, Lambert L, Orr-Gonzalez S, Herrod J, Robbins YL, et al. *Grammomys surdaster*, the natural host for *Plasmodium berghei* parasites, as a model to study whole-organism vaccines against malaria. Am J Trop Med Hyg. 2017;96:835–41.
48. Schaer J, Perkins SL, Decher J, Leendertz FH, Fahr J, Weber N, et al. High diversity of West African bat malaria parasites and a tight link with rodent *Plasmodium* taxa. Proc Natl Acad Sci USA. 2013;110:17415–9.
49. O'Donnell AJ, Rund SS, Reece SE. Time-of-day of blood-feeding: effects on mosquito life history and malaria transmission. Parasit Vectors. 2019;12:301.
50. Desowitz R, Barnwell J. *Plasmodium berghei*: deep vascular sequestration of young forms in the heart and kidney of the white rat. Ann Trop Med Parasitol. 1976;70:475–6.

Publisher's Note

Paper #6 (2021b): Mistimed malaria parasites re-synchronize with host feeding-fasting rhythms by shortening the duration of intra-erythrocytic development

Received: 22 June 2021 | Revised: 8 November 2021 | Accepted: 9 November 2021

DOI: 10.1111/pim.12898

Parasite Immunology WILEY

INVITED REVIEW

Mistimed malaria parasites re-synchronize with host feeding-fasting rhythms by shortening the duration of intra-erythrocytic development

Aidan J. O'Donnell[1] | Megan A. Greischar[2] | Sarah E. Reece[1]

[1]Institute of Evolutionary Biology, and Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh, UK

[2]Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York, USA

Correspondence
Aidan O'Donnell, Institute of Evolutionary Biology, and Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh, UK.
Email: Aidan.ODonnell@ed.ac.uk

Funding information
This work was supported by Wellcome (grant nos. 202769/Z/16/Z; 204511/Z/16/Z), the Royal Society (grant no. URF\R\180020), and funding from the College of Agriculture and Life Sciences, Cornell University.

Abstract

Aims: Malaria parasites exhibit daily rhythms in the intra-erythrocytic development cycle (IDC) that underpins asexual replication in the blood. The IDC schedule is aligned with the timing of host feeding-fasting rhythms. When the IDC schedule is perturbed to become mismatched to host rhythms, it readily reschedules but it is not known how.

Methods: We intensively follow four groups of infections that have different temporal alignments between host rhythms and the IDC schedule for 10 days, before and after the peak in asexual densities. We compare how the duration, synchrony and timing of the IDC differs between parasites in control infections and those forced to reschedule by 12 hours and ask whether the density of parasites affects the rescheduling process.

Results and conclusions: Our experiments reveal parasites shorten the IDC duration by 2–3 hours to become realigned to host feeding-fasting rhythms with 5–6 days, in a density-independent manner. Furthermore, parasites are able to reschedule without significant fitness costs for them or their hosts. Understanding the extent of, and limits on, plasticity in the IDC schedule may reveal targets for novel interventions, such as drugs to disrupt IDC regulation and preventing IDC dormancy conferring tolerance to existing drugs.

KEYWORDS
asexual replication, biological rhythm, circadian, fitness, intra-erythrocytic development cycle, periodicity, *Plasmodium*

1 | INTRODUCTION

Biological rhythms enable organisms to undertake activities at the time of day they are best undertaken. For example, cycles of activity and rest occur in relation to the day-night cycles driven by the 24 hourly rotation of the earth, in the manners that minimize exposure to predators or harsh environmental conditions or maximize mating opportunities.[1-3] Biological rhythms are also important in the context of infections.[4,5] Many aspects of host immunity oscillate with a daily rhythm[6,7] and diverse parasites align activities to daily rhythms in transmission opportunities,[8] resource availability[9] and host defences.[10] For example, transmission stages of filarial nematodes, including *Wuchereria bancrofti*, migrate from host tissues to the peripheral capillaries in a periodic manner to coincide

with daily rhythms in the biting activity of their mosquito vectors[8] and *Schistosoma* spp. cercariae emerge from their intermediate snail host in the early morning or evening, depending on whether the next host in their lifecycle is nocturnal or diurnal.[11,12] Scheduling transmission activities extend beyond coordinating with vector and host rhythms; daily rhythms in the environment also impose opportunities and constraints on transmission. For example, the sporulation of oocysts produced by *Isospora spp*. is reduced by UV light exposure and so, oocysts are shed in the host's faeces in the afternoon and evening.[13,14] Once inside a host, parasites are subjected to the full gamut of the host's rhythms, including cellular and molecular processes, physiologies and metabolism and its behaviours. For example, host circadian clocks control cellular processes that influence the success of viral entry into cells and dissemination through tissues, for SARS-CoV-2,[15] hepatitis B[16] and influenza.[17] Viruses do not appear to have rhythms in their own activities but instead may manipulate host rhythms to facilitate replication.[5,17] Daily rhythms in the feeding-fasting cycles of hosts appear to drive periodicity in the gene expression patterns of *Trypanosoma brucei* [9] and *Schistosoma mansoni*,[18] as well as setting the timing of blood stage replication by *Plasmodium* spp. (malaria parasites).[19-21]

Intuition suggests the diverse rhythms documented in parasites should enhance fitness via between-host transmission and/or within-host survival. Across all pathogenic organisms, rhythms in malaria parasites are currently the best understood; from evolutionary and ecological perspectives to their molecular underpinnings.[4] Malaria parasites exhibit rhythms lasting a multiple of 24 hours in the intra-erythrocytic development cycle (IDC) which underpins asexual replication in the vertebrate host's red blood cells.[22,23] Specifically, malaria parasites develop synchronously throughout the IDC and burst to release progeny at a particular time of day which generates fever with a 24, 48, or 72 hour periodicity that characterizes malaria infection by different *Plasmodium spp*. For *Plasmodium chabaudi*, over 57% of the transcriptome is rhythmic, the IDC exhibits 24h periodicity and culminates at the end of the hosts feeding period.[19,20,24,25] Whilst the timing of host feeding-fasting and metabolic rhythms are ultimately determined by the host's clock, the host's canonical transcription-translation feed-back loop (TTFL) clock does not directly affect the IDC schedule.[19] Instead, the timing of transitions between the developmental stages of the IDC directly follows feeding-fasting rhythms, with rhythmicity in the amino acid isoleucine fulfilling the criteria to act as a time cue.[26] Coordinating the IDC schedule with host rhythms is important for parasite fitness. When the timing of the IDC schedule is out of synchrony with the host, parasites suffer losses in the number of both asexually replicating stages and sexual transmission stages[27-29] are more vulnerable to antimalarial drug treatment,[30] and gene expression patterns underpinning key cellular processes are significantly altered.[25] Thus, *P. chabaudi's* IDC schedule allows parasites to maximally exploit rhythmicity in the resources they require from the host's food.[20] Conveniently, this schedule also ensures the maturation of sexual stages coincides with the time-of-day vectors forage for blood.[31]

How the IDC schedule is aligned with host rhythms is mysterious. Parasites may simply be intrinsically arrhythmic yet benefit from rhythms imposed upon them by the rhythms of hosts/vectors. For example, perhaps mistimed IDC stages starve and die because host rhythms create an environment in which only certain stages survive at certain times of day. Most evidence suggests that malaria parasites (at least in large part) control their timing.[4,25,32-34] This includes observations that *P. falciparum* can undergo dormancy during the IDC to survive antimalarial drug treatment,[35] *P. chabaudi* controls its IDC duration via the gene, Serpentine Receptor Ten[25] and both *P. chabaudi* and *P. falciparum* use a cue with a daily rhythm (isoleucine) to break IDC dormancy.[34,36] A key step in differentiating between the relative contributions of traits encoded by the genes of hosts vs parasites is to search for time-keeping mechanisms in parasites. The components of clocks driven by TTFLs have been identified in the fungal pathogen *Botrytis cinerea* and its clock is used to schedule the expression of virulence genes.[10,37] However, there is little homology in the genes underpinning canonical circadian oscillators across divergent taxa, complicating the search for 'clock genes' in novel organisms.[38-40] Further, parasites may keep time with simpler 'reactionary' strategies rather than circadian clocks (which confer the additional abilities of temperature compensation and anticipation), or via oscillators that pre-date the TTFL.[4,41,42] Gene expression rhythms in trypanosomes and malaria parasites do fulfil some of the phenotypic criteria of endogenous TTFL-driven oscillators.[9,25,29,32,33,43]

Given the importance of timing the IDC schedule correctly coupled with parasites' likely ability to keep time, it is not surprising that when the timing of the IDC schedule is perturbed, parasites readily reschedule. For example, *P. chabaudi* recovers from a 12-hour mismatch to the host's feeding-fasting rhythm within 5–7 IDCs.[19,29] During natural infections, parasites may benefit from a time-keeping ability if egress from the liver to initiate blood stage replication occurs asynchronously or at a different time of day to optimal for IDC stages. Here, we ask how plasticity (flexibility) in *P. chabaudi's* IDC schedule allows malaria parasites coordinate with host rhythms. Following a 12-hour mismatch to host rhythms, we test whether rescheduling of the IDC involves parasite development speeding up or slowing down, and we examine the consequences of rescheduling for synchrony, timing and replication dynamics. Determining how the IDC schedule responds to mismatch required tracking infections over at least 7 days with samples collected every few hours. However, after several days of intensive sampling regimes, host rhythms become perturbed which has knock-on consequences for parasite rhythms.[44] To overcome this issue, we set up multiple cohorts by infecting mice a day apart such that mice in each cohort were sampled simultaneously only over a 24–28 hour window, with each cohort contributing data for a different day post infection.

We made no *a priori* predictions for how the IDC should reschedule due to contradictory observations in the literature, including that (i) closely related species have shorter (and asynchronous) IDC durations (22–23 hours for *P. berghei* and 18h for *P. yoelii* [45,46]) suggesting faster IDCs are biologically possible; (ii) the avian malaria *P. cathemerium*, appears to extend or reduce

its IDC duration in response to different perturbations of host rhythms[47,48]; (iii) the IDC is arrested in response to the loss of a putative timing cue[26] suggesting mismatched parasites only need a 12-hour pause to get back on time; and (iv) a 12-hour mismatch means that the same amount of time must be recovered by either speeding up or slowing down, so taking (ii) and (iii) together, different parasites within and between infections may adopt opposite strategies, as suggested for *P. brasilianum*.[43] Changes in the duration of the IDC could affect overall asexual replication in a number of non-mutually exclusive ways. Intuitively, a shorter IDC should lead to faster replication over the course of infection, but this depends on whether speeding up comes with a cost of fewer progeny per parasite (ie fewer merozoites per schizont), or if lower 'quality' progeny arise from a mismatch to nutritional resources or less time overall to garner resources. Understanding the extent of, and limits on, plasticity in the IDC schedule is important because asexual replication is responsible for the severe symptoms of malaria and fuels the production of sexual transmission stages and conferring tolerance to antimalarials.[4,35]

2 | MATERIALS AND METHODS

We carried out a large-scale experiment to investigate how the IDC reschedules to regain synchrony following different kinds of perturbation to the host's feeding-fasting rhythm ('rescheduling'), and a smaller repeat study to test whether parasite density influences the rescheduling process ('dose dependency').

2.1 | Hosts and parasites

Hosts were either wild type (WT) C57BL/6J strain or *Per1/2*-null clock-disrupted mice previously backcrossed onto a C57BL/6J background for over 10 generations. *Per1/2*-null mice lack genes (Period1 and Period2) that are integral for a functional core (TTLF) clock and as a result, are behaviourally arrhythmic (including feeding-fasting patterns) when housed in constant darkness.[19,49,50] Mice were mixed sexes, 8–10 weeks old, housed at 21°C, and given a standard RM3 pelleted diet (801700, SDS, UK) with unrestricted access to drinking water supplemented with 0.05% para-aminobenzoic acid.[51] All mice were allowed 2 weeks to acclimatize ('entrain') to their respective feeding-fasting/light-dark rhythms before being infected. *P. chabaudi* (clone DK) parasites were injected intravenously at a dose of 1×10^6 parasitized RBCs for the rescheduling experiment or at either 1×10^5 (low dose) or 1×10^7 (high dose) parasitized RBCs for the test of dose dependency. To reduce any potential donor effects, inoculum consisted of a pooled mix of three donor mice given to all treatment groups within each cohort. All procedures were carried out in accordance with the UK Home Office regulations (Animals Scientific Procedures Act 1986; SI 2012/3039) and approved by the ethical review panel at the University of Edinburgh.

2.2 | Experimental designs

For the rescheduling experiment, wild type (WT) and *Per1/2*-null mice were assigned to 4 treatment groups (n = 16 per group; Figure 1). The two WT treatments differed by their lighting regime (lights on 20:00–08:00 GMT (DL) and lights on 08:00–20:00 GMT (LD)) and were each provided with all-day access to food (*ad libitum*). Mice in these groups followed their usual nocturnal feeding rhythms and fed primarily in their dark phases (08:00–20:00 GMT for the DL group and 20:00– 08:00 GMT for the LD group). The two groups of *Per1/2*-null mice were housed in constant darkness (DD, with dim red LED) and provided with either a time-restricted feeding diet (TRF) in which food was only available 21:00 to 09:00 GMT (analogous to the feeding window of the WT LD treatment) or was allowed all-day access to food. Due to their arrhythmic behaviour, mice in the latter group feed continually throughout the 24h day.[19] Note, TRF protocols differ from dietary restriction in that there are no weight loss implications of TRF (*Per1/2*-null TRF mean ± SEM weight loss (g) for the 2 week entrainment period before infection = 0.1 ± 0.57).

Each of the four treatment groups were split into four cohorts (n = 4 mice per cohort per treatment) and infected with a synchronous population of ring stage parasites originating from donors housed in DL. This generated treatment groups in which parasites were matched to host feeding rhythms (WT matched); mismatched to host feeding rhythms and must reschedule by ~12 hours (WT mismatched and *Per1/2*-null TRF); and infecting hosts without host feeding rhythms (*Per1/2*-null all-day fed). Cohorts were infected in a staggered design with the first cohort infected on day 5 followed by the other cohorts on days 4, 3 and 2. As a result, at any sampling time point, infections within each treatment group span 4 consecutive days post infection. Each cohort can therefore be concatenated to form a time series spanning multiple days.

To test whether the main experiment revealed general patterns for rescheduling or if the process depends on parasite density we compared how many IDC were required for parasites in WT mismatched infections initiated with two different doses (1×10^5 and 1×10^7 infected RBCs) to reschedule to the host's feeding-fasting rhythm. Infections (n = 5 per cohort per dose) were initiated with the same staggered design for 4 cohorts as above and sampled every 4 hours for 32 hours from 08:00 GMT spanning day 2–6 PI.

2.3 | Sampling and data collection

For the rescheduling experiment, mice were sampled at 4-hourly intervals over two windows; for 28h to generate a pre-peak window time series spanning days 2–6 PI, and for 24h to generate a post-peak window time series spanning days 7–10 PI. The sampling regimes were set such that each cohort overlapped with the preceding/subsequent cohorts in terms of hours post infection (hpi). For the pre-peak time series, each cohort overlapped by 2 sampling points, by a single sample overlap in the post-peak time series, and by 3 sampling points for the dose experiment.

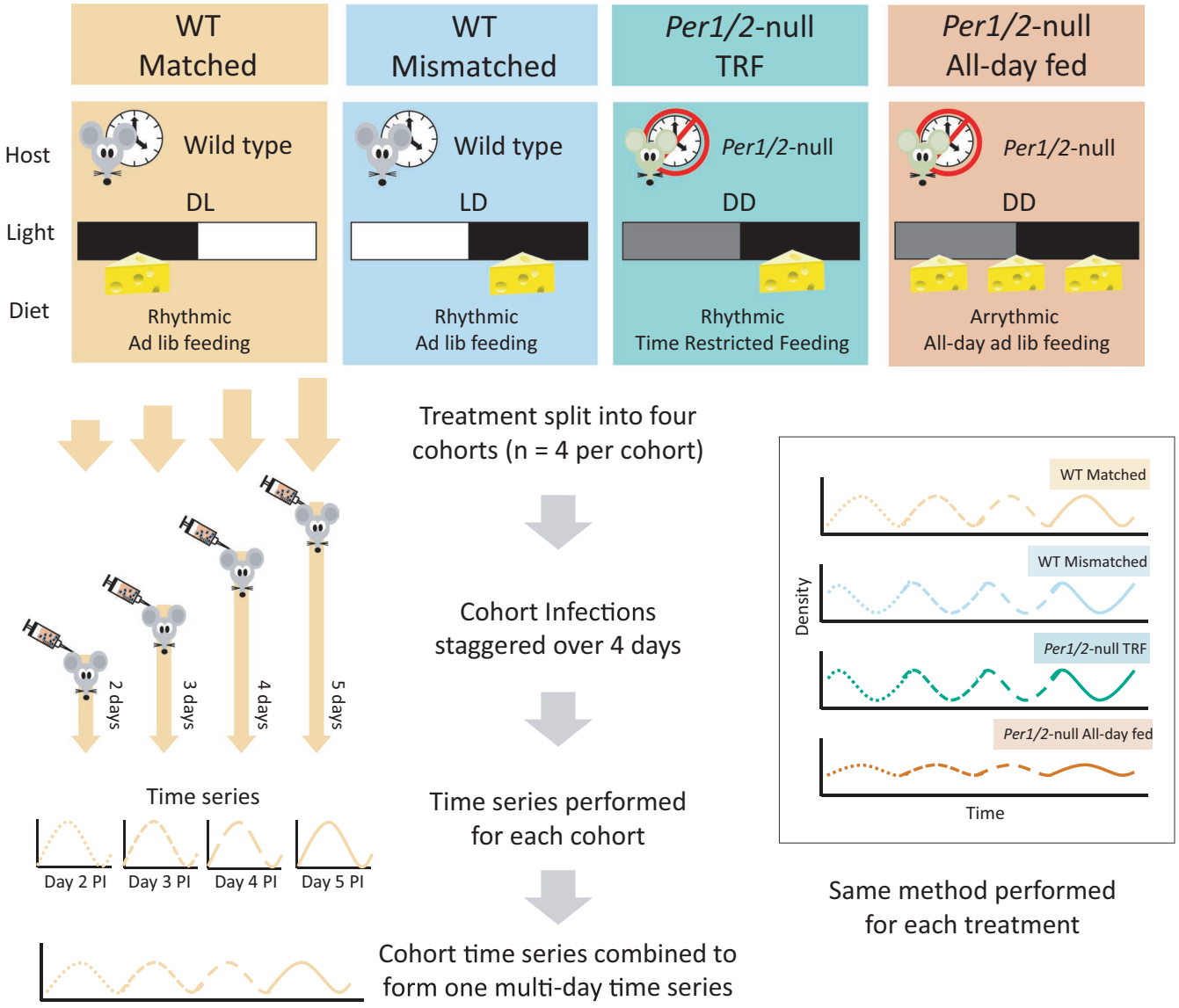

FIGURE 1 Experimental design. Four treatment groups were created from WT (C57BL/6J) or TTFL-clock-disrupted *Per1/2*-null mice housed in a standard (LD) or reversed (DL) photoschedule, or constant darkness (DD), and given constant access to food (*ad lib* diet) or were fed with a time-restricted diet (food available for only 12 hours per day; TRF). Mice from each of these groups were allocated to 4 cohorts (n = 4 each cohort per treatment). Cohorts within each treatment were infected over 4 subsequent days with ring stage parasites from WT donors entrained to a DL photoschedule. Thus, with respect to host feeding-fasting rhythms, parasites in the matched treatment entered hosts in the same phase as their donor hosts (WT matched), parasites in the mismatched and TRF groups were ~12 hours out of phase to their hosts and must reschedule (WT mismatched & *Per1/2*-null TRF), and parasites in the all-day fed treatment entered arrhythmic hosts in which IDC rhythms become dampened (*Per1/2*-null all-day fed). All mice in all four cohorts were sampled on the same calendar day, every 4h for 28h, to cover the pre-peak window of infections which spans days 2 to 6 post infection (PI), and cohorts 1–3 were sampled again (4h sampling for 24h) after a 3 day break to generate a post-peak dataset covering days 7–10 PI. The time series for the cohorts with each treatment group were concatenated to generate pre-peak and post-peak time series for period estimates, whereas other rhythm parameters were estimated from individual infections

These overlaps allowed us to determine if infections within each treatment were repeatable enough across cohorts to concatenate their data for some of the analyses. All mice contributed samples throughout the pre-peak window for the main experiment (n = 4 per cohort) and the dose comparison (n = 5 per cohort). Five mice (2 from the *Per1/2*-null TRF group and 3 from the *Per1/2*-null all-day fed group) were withdrawn from the experiment due to severe malaria symptoms following the pre-peak window. This reduced the sample sizes in the *Per1/2*-null TRF group to 3 for cohorts covering days 7–8 and 9–10 PI and in the *Per1/2*-null all-day fed group the cohorts covering days 8–9 and days 9–10 PI were reduced to 3 and 2 respectively. At each sampling point, a thin blood smear was taken to assess IDC stage distribution (from the proportion of parasites at each IDC stage in each smear) and RBC densities per ml were measured by flow cytometry (Z2 Coulter Counter, Beckman Coulter) immediately after sample collection. Blood smears were

stained with 10% Giemsa for 12mins and IDC stages quantified based on parasite size, number of nuclei and the appearance of haemozoin, and summed to estimate total parasites.[19,20] Ring stage density per ml of blood was obtained from the product of the proportion of rings and RBC density.

2.4 | Data analysis

Parasite densities at time-points in which cohorts overlap were log-transformed and compared between cohorts using either linear mixed-effect models with mouse identity fitted as a random effect (pre-peak and dose-dependence datasets, due to multiple overlaps) or with GLMs (post-peak dataset, due to one overlap). Parameters of rhythmicity (amplitude, phase, period) were determined using a maximum entropy spectral analysis (MESA).[52] Infections for which MESA could successfully fit a rhythm between the period limits of 18–34 hours were classed as rhythmic. MESA was chosen because it is robust against baseline trends and large differences in amplitude across time that are characteristic of parasite density dynamics. Verification of MESA outputs was performed using Fast Fourier Transform Non-linear Least Squares (FFT-NLLS), Lomb-Scargle and MetaCycle (Meta2d).[53] Before rhythmicity analysis, ring densities were log-transformed to reduce the exponential increase exhibited during infections. For the period analyses, additional baseline detrending via kernel smoothing was also performed (detrending was not necessary for amplitude and phase analyses). Rhythm amplitude and phase were determined from the time series data from each infection individually (time series length: pre-peak window = 28 hour, post-peak window = 24 hour). Amplitude is a unit-less measure (denoted numerically between zero and one) representing the relative difference between maximum and minimum of an oscillation and was analysed with generalized linear models (GLMs). Phase represents peak timing of the oscillation (ie peak ring density) and was analysed with Bayesian circular GLMs. Period measures are best determined from longer time series with multiple cycles and therefore were calculated from datasets generated by averaging replicates within cohorts at each time point. Before period analysis, rhythmicity of the concatenated dataset was verified using the BD2 eJTK method. Parasite densities across hpi were compared between treatments in each infection window using linear mixed-effect models with mouse identity nested within cohort as a random effect. RBC loss and weight loss were calculated for each cohort by subtracting the RBC/weight at the end of the time series from the beginning and were analysed using GLMs. For all models, to avoid overfitting due to small sample sizes 'Akaike information criterion-corrected' (AICc) values were calculated, and a change in 2 AICc (ΔAICc = 2) was chosen to select the most parsimonious model. Rhythmicity analysis, MESA, FFT-NLLS and Lomb-Scargle analyses were performed with Biodare2 (https://biodare2.ed.ac.uk/)[54] and all other analyses, including the Metacycle rhythmicity analysis, were performed with R v. 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

3 | RESULTS

3.1 | Concatenating cohorts

To confirm that cohorts within each treatment are repeatable enough to represent longitudinally sampled infections, we compared parasite densities at the hours post infection (hpi) for which consecutive pairs of cohorts overlapped (Figure 2). For both the pre- and post-peak window of the infections, incorporating cohort into the models did not improve model fits indicating that densities at these time points did not vary significantly between cohorts (Supplementary Information (SI) Table 1). Thus, we proceed with using data concatenated across cohorts for estimations of period and analyses of density dynamics. Whereas other characteristics of rhythms (amplitude, phase) can be calculated from the short time series for each individual infection.

3.2 | IDC rhythms during rescheduling

We focus on ring stages as a marker for the IDC schedule, as is usual for studies of *P. chabaudi* IDC rhythms[19,20,25,28,29] (Figure 3; and Figure S1). In the pre-peak window (days 2–6 PI) all infections exhibited rhythmicity in ring stage density except for a single infection in the WT matched treatment (from the days 2–3 cohort). In the post-peak window (days 7–10 PI), 9/12 infections in both WT treatments were rhythmic, 8/10 infections were rhythmic in the *Per1/2*-null TRF group and 6/9 infections were rhythmic in the *Per1/2*-null all-day fed group. All treatment groups in the concatenated time series were cyclic according to multiple approaches for assessing rhythmicity (Table 1). Because rhythmicity parameters can only be estimated for rhythmic infections, the non-rhythmic infections were excluded for estimates of period, amplitude and phase.

3.2.1 | IDC duration (Period)

During the pre-peak window, the concatenated time series reveal that periods were 1–2 hours shorter in the treatment groups with rescheduling parasites (WT mismatched = 21.30h, *Per1/2*-null TRF = 22.56h) compared with infections matched to host feeding rhythms (WT matched = 23.40h; Table 1; Figure S2). Infections in hosts without feeding rhythms were also short (*Per1/2*-null all-day fed = 22.5h). The short period in rescheduling infections is evident by the five full peaks observed throughout the time series, whilst the WT matched infections had yet to reach the apex of peak five (Figure 3).

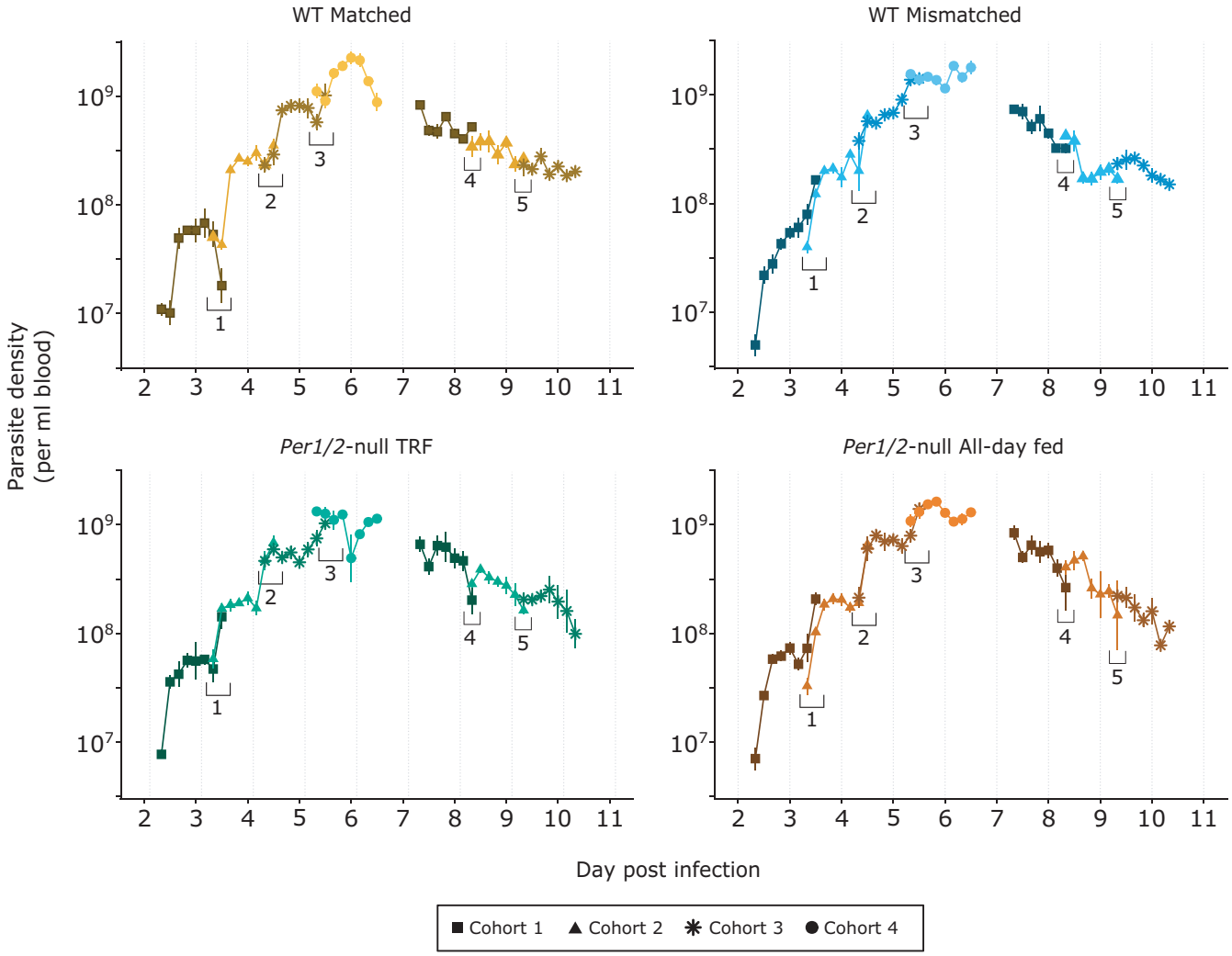

FIGURE 2 Mean ± SEM parasite density (per ml blood). Each cohort (represented by differing point shape and colour gradient) was comprised of replicate infections sampled over subsequent days post infection. Time points in which samples occurred at the same age of infection for each consecutive pair of cohorts are indicated by numbered brackets. Overlaps 1–3 occurred in the pre-peak window and each consisted of 2 time points, whereas overlaps 4–5 each had single time point and occurred in the post-peak window. Mice were either WT (C57BL/6J) or clock-disrupted *Per1/2*-null mice with parasites that were matched to the host's feeding-fasting rhythm (WT matched), forced to reschedule to align with the host's feeding-fasting rhythm (WT mismatched & *Per1/2*-null TRF), or experienced arrhythmic hosts (*Per1/2*-null all-day fed). n = 4 infections per cohort for all groups in the pre-peak window. For the post-peak window, n = 4 for WT groups, n = 3–4 for *Per1/2*-null TRF, and n = 2–4 for the *Per1/2*-null all-day fed group

Post-peak model fits were generally poorer compared with pre-peak model fits but suggest that period remained close to 24 hours in WT matched infections (23.34h), lengthened by 3 hours to reach approximately 24 hours in the WT mismatched group (24.04h), and infections in *Per1/2*-null hosts also extended by 3–5 hours to exceed 24 hours (*Per1/2*-null TRF =26.88h, *Per1/2*-null all-day fed =27.90h; Table 1; Figure S3).

3.2.2 | IDC synchrony (amplitude)

We estimated rhythm amplitude (change between peak and trough for ring density) of each individual infection from its 28/24 hour time series (pre-peak/post-peak windows). During the pre-peak window of infections (days 2–6 PI), amplitude is best described by the model containing only treatment as a main effect (ΔAICc = 0, AICc weight = 0.94; Table S2). Specifically, parasites already coordinated with host feeding-fasting rhythms (WT matched) had rhythm amplitudes ~50% higher than parasites in treatments causing rescheduling (WT mismatched and *Per1/2*-null TRF) and in arrhythmic hosts (Figure 4a; Figure S4a; amplitude mean ± SEM: WT matched = 0.75 ± 0.03, WT mismatched = 0.50 ± 0.03, *Per1/2*-null TRF = 0.55 ± 0.04, *Per1/2*-null all-day fed = 0.54 ± 0.03). Incorporating day PI reduced model fits (ΔAICc = 5.49, weight = 0.06; Table S2) indicating that rhythm amplitude did not change significantly during the pre-peak window.

Amplitude also varied during the post-peak window of infection (days 7–10 PI) in a manner best explained by additive effects of day PI and treatment (ΔAICc = 0, weight = 0.99; Table S2). Specifically, WT matched infections had the highest amplitude, 55%

TABLE 1 Rhythmicity analysis and measures of IDC period calculated from representative datasets (log^{10} ring stage density averaged across replicate infections contributing to each time point). For the rhythmicity analysis empirical-JTK was performed and Benjamini Hochberg (BH) corrected *p* values are presented. For the period analysis, each dataset was analysed using Maximum Entropy Spectral Analysis (MESA, in bold) and results verified with Fast Fourier Transform Non-linear Least Squares (FFT-NLLS), Lomb-Scargle and Metacycle (Meta2d). Each period estimates is accompanied by the model's goodness of fit (GoF; for which values close to zero indicate better fits), or for Meta2d, the BH corrected p value is appropriate. For the pre-peak window, period was calculated using a 102h time series including four to five IDC cycles, and for the post-peak window, period was calculated using a 72h dataset representing three IDC cycles. In both datasets mice were sampled every 4h

		IDC period (hours)							
		MESA		*FFT-NLLS*		*Lomb-Scargle*		*Meta2d*	
	Rhythmicity	Period	GoF	Period	GoF	Period	GoF	Period	*p*
Pre-peak window									
WT matched	<0.0001	**23.4**	**0.29**	23.59 ± 0.63	0.32	23.58	0.28	23.84	<.0001
WT mismatched	<0.0001	**21.3**	**0.38**	21.38 ± 0.69	0.41	21.36	0.38	20.89	<.0001
Per1/2-null TRF	<0.0001	**22.56**	**0.38**	22.45 ± 0.66	0.36	22.44	0.31	22.94	<.0001
Per1/2-null all-day fed	<0.0001	**22.5**	**0.26**	22.60 ± 0.59	0.29	22.6	0.25	23.03	<.0001
Post-peak window									
WT matched	0.016	**23.34**	**0.67**	23.21 ± 2.41	0.58	23.32	0.54	23.77	<.0001
WT mismatched	0.001	**24.04**	**0.59**	24.00 ± 2.31	0.61	24.02	0.53	24.19	<.0001
Per1/2-null TRF	0.003	**26.88**	**0.53**	27.30 ± 3.01	0.55	27.3	0.52	27.52	<.0001
Per1/2-null all-day fed	0.016	**27.9**	**0.55**	30.04 ± 4.91	0.65	29.98	0.61	30.7	<.0001

higher than rescheduling infections (WT mismatched and *Per1/2*-null TRF) and ~200% higher than parasites in arrhythmic hosts (Figure 4a; amplitude mean ± SEM: matched = 0.48 ± 0.05, WT mismatched = 0.33 ± 0.06, *Per1/2*-null TRF = 0.30 ± 0.04, *Per1/2*-null all-day fed = 0.23 ± 0.03). During the post-peak window, amplitudes decreased from an average of 0.45 ± 0.04 on day 7 PI to 0.30 ± 0.05 on day 10 PI (Figure 4a; Figure S4a).

3.2.3 | IDC timing (phase)

We estimated the peak timing of ring density ('phase marker') for each individual infection from their 28/24 hour time series (pre-peak/post-peak windows). During the pre-peak window of infections, phase is best explained by the interaction between treatment and day PI (Table S3). Around day 2 PI, the mean phases (hour GMT ± SD) are 19.58 ± 0.28 for WT matched infections, 14.95 ± 0.38 for WT mismatched, 16.07 ± 0.15 for *Per1/2*-null TRF and 16.63 ± 0.07 for *Per1/2*-null all-day fed (Figure 4b,c; Figure 4b). For the WT matched infections, this timing aligns with the end of the host's feeding period (dark period) and donor infections, illustrating that these parasites have maintained their IDC rhythm. Peak phase in the rescheduling (WT mismatched and *Per1/2*-null TRF groups) and *Per1/2*-null all-day fed groups had deviated by 3–5 hours, suggesting that whilst they were still mismatched to their new host's feeding-fasting rhythm, rescheduling was underway.

As infections progressed, the phase of WT matched infections varied little, but the phase of rescheduling parasites (WT mismatched and *Per1/2*-null TRF) diverged by approximately 10 hours to become aligned to host feeding-fasting rhythms by day 6 PI (mean phase hour GMT ± SD: WT matched = 17.54 ± 0.18, WT mismatched = 6.26 ± 0.28, *Per1/2*-null TRF = 8.14 ± 0.15). Infections in the *Per1/2*-null all-day fed group also deviated from the phase of the WT matched group with peak ring density at 10.92 h ± 0.46 GMT, five hours earlier than WT matched groups and 3–5 hours later than rescheduling groups. Overall, during the pre-peak window, the mean rate of phase change for rescheduling infections was −2.77 ± 0.90 hours per day and a slower mean rate of phase change for the *Per1/2*-null all-day fed group of −1.90 ± 0.38 hours per day.

During the post-peak window, the phase of peak ring density is also best explained by the interaction between treatment and days PI (Table S3). However, unlike in the pre-peak window, phase change is not directional for all groups throughout the post-peak window. Specifically, on day 7 PI the rescheduling infections and *Per1/2*-null all-day fed infections peak at a similar time, 7–8 hours earlier than WT Matched infections (Figure 4b,c; Figure S4b; mean phase hour GMT ± SD: WT matched = 19.52 ± 0.04, WT mismatched = 11.79 ± 0.36, *Per1/2*-null TRF = 12.80 ± 0.78, *Per1/2*-null all-day fed = 12.97 ± 2.37). Phase became later in all groups by days 8–9 but patterns diverged by day 10 PI. Across days 7–10 PI, *Per1/2*-null TRF and *Per1/2*-null all-day fed infections peaked at a similar time to WT matched infections (mean phase hour GMT ± SD: WT matched = 17.71 ± 0.40, *Per1/2*-null TRF = 18.43 ± 0.05, *Per1/2*-null all-day fed = 18.34 ± 0.18), but the peak of WT mismatched infections became 9 hours earlier (9.04h ± 0.89 GMT) between days 8 and 10 PI.

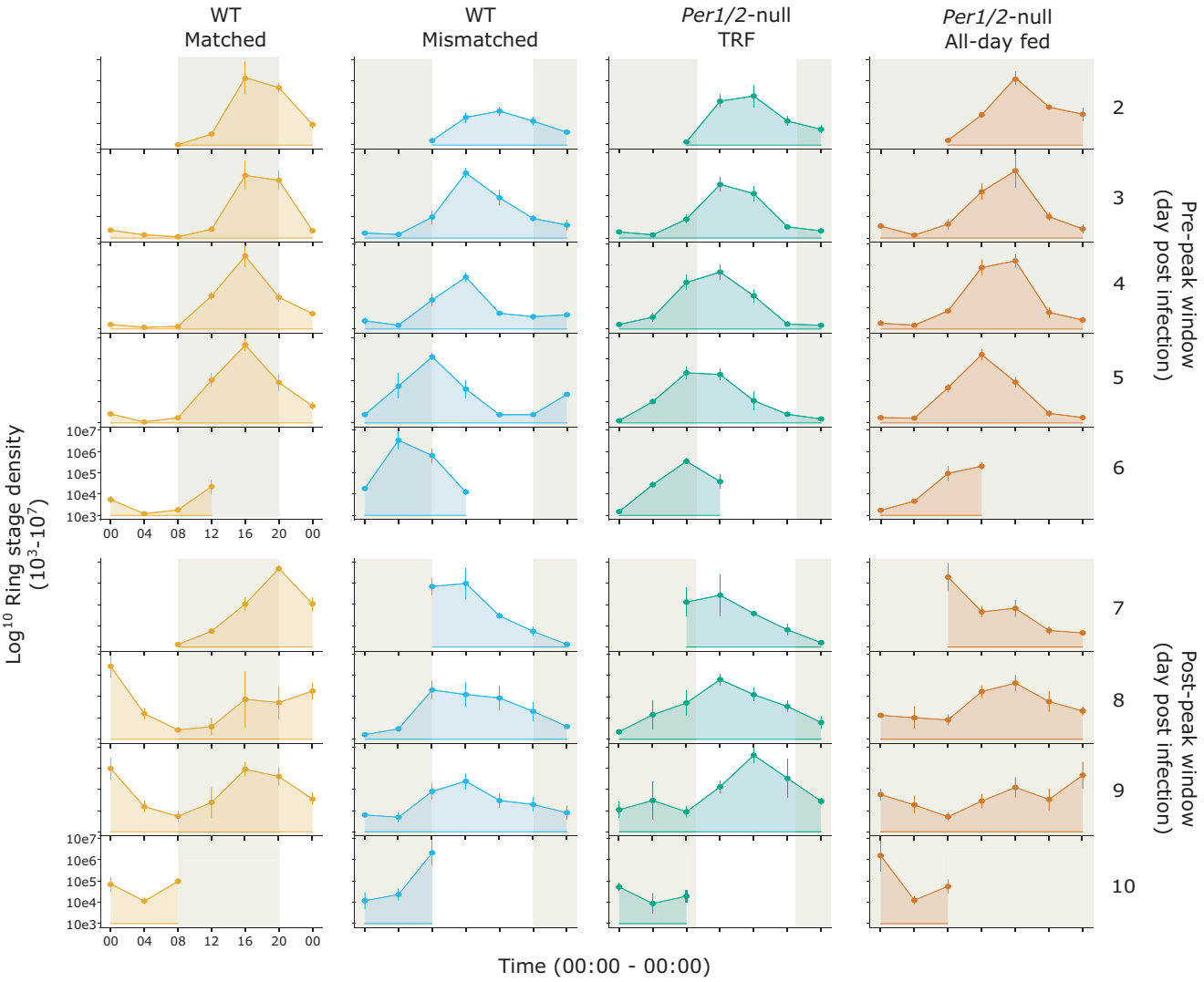

FIGURE 3 Mean ± SEM ring stage parasite density (per ml blood). Mice were either WT (C57BL/6J) or clock-disrupted *Per1/2*-null mice with parasites that were matched to the host's feeding-fasting rhythm (WT matched), forced to reschedule to align with the host's feeding-fasting rhythm (WT mismatched & *Per1/2*-null TRF) or experienced arrhythmic hosts (*Per1/2*-null all-day fed). Shading represents the windows in which hosts fed and axes scales are identical across all plots. All infections including those without significant rhythms are included in the Mean ± SEM calculations: n = 4 infections per cohort for all groups in the pre-peak window. For the post-peak window, n = 4 for WT groups, n = 3–4 for *Per1/2*-null TRF, and n = 2–4 for the *Per1/2*-null all-day fed group.

3.3 | Infective dose and rescheduling

In our second experiment, we compared the rates of IDC rescheduling by parasites in WT mismatched infections initiated with doses two orders of magnitude apart (10× higher and lower than the main rescheduling experiment). Asexual densities reflect the different infective doses, with low dose infections achieving a lower cumulative density than high dose infections (mean total cumulative parasite density ± SEM ($\times 10^{10}$): low = 6.19 ± 0.14, high = 22.80 ± 0.08), and daily cumulative densities are best explained by the model with an interaction of days PI and dose (ΔAICc = 0, weight = 1; Figure 5a). Comparison of the overlaps revealed only minor (~3%) cohort differences between some infections in overlap 1 (Table S4), thus in concordance with the main rescheduling experiment, we concatenate cohorts to generate a single time series for each dose.

Resolution on the IDC schedule is low at the start of infections for the low dose because the fewer parasites that are used to initiate infections, the fewer that are observed for staging. Despite noisy date between days 2 and 3 PI in the low dose group, all infections exhibited very similar IDC rhythms during rescheduling (Figure 5). Once rescheduled to align with host feeding-fasting rhythms, ring densities peak at the end of the feeding window (ie the right-hand side of the shaded regions in Figure 5a,b) and both dose groups achieved this timing between days 5 and 6 PI. Specifically, mean phase hours (GMT) ± SD on day 6 PI were 6.36 ± 0.5 and 6.65 ± 0.21 for the low and high dose groups. The concatenated time series reveal that both low and high dose

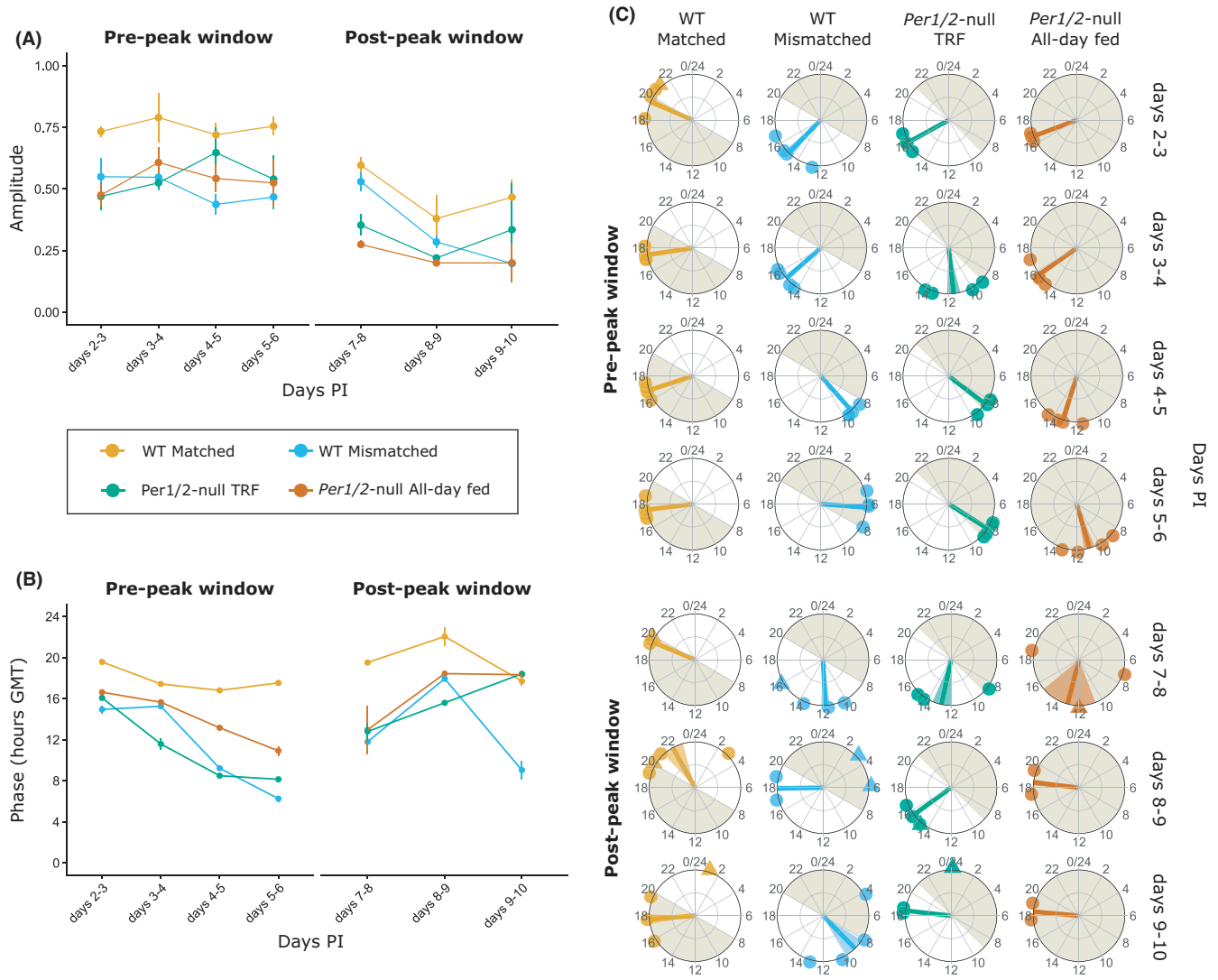

FIGURE 4 Mean ± SEM (A) ring stage amplitude and (B & C) peak ring stage phase calculated from ring stage density data for all rhythmic infections using a Maximum Entropy Spectral Analysis (MESA). In (C), mean peak ring stage phase is represented by a line with circular SD in shading. Circles and triangles represent phase estimates from infections classed as rhythmic and non-rhythmic, respectively (the latter are omitted from (B) and do not influence mean ± SEM/SD but are included for completeness). Mice were either WT (C57BL/6J) or clock-disrupted *Per1/2*-null mice with parasites that were matched to the host's feeding-fasting rhythm (WT Matched), forced to reschedule to align with the host's feeding-fasting rhythm (WT Mismatched & *Per1/2*-null TRF) or experienced arrhythmic hosts (*Per1/2*-null all-day fed). Shading represents the windows in which hosts fed. N = 4 infections per cohort for all groups in the pre-peak window apart from WT matched (n = 3–4/cohort). For the post-peak window, n = 2–4 for WT groups, n = 2–3 for *Per1/2*-null TRF, and n = 2 for the *Per1/2*-null all-day fed group

infections have periods of less than 24 hours (low dose = 21.52, high dose = 22.64). Further, both phase and amplitude do not differ (from day 3PI) between doses (amplitude is best explained by the null model (ΔAICc = 0, weight = 0.58; Table S5), and phase is best explained by the model containing days PI (Table S6).

3.4 | Consequences of rescheduling for parasites and hosts

The design of the main rescheduling experiment enables us to examine whether different phase relationships between parasite and host rhythms influence the densities of asexual stages achieved over infections and the severity of symptoms experienced by hosts.

3.4.1 | Parasite performance

Parasite densities during both the pre- and post-peak windows are best explained by day PI only (pre: ΔAICc = 0, weight = 0.997; post: ΔAICc = 0, weight = 0.997; Table S7) only. Including treatment reduced model fits (pre: ΔAICc = 11.70, weight = 0.003; post: ΔAICc = 11.53, weight = 0.003; Table S7), indicating that parasite densities during infections do not differ between treatments (Figure 6a,b).

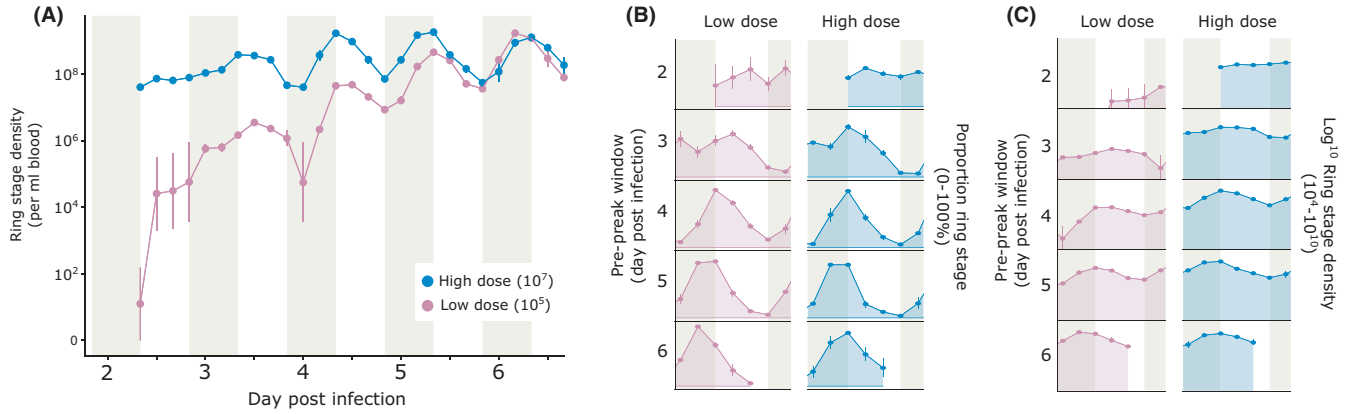

FIGURE 5 Mean ± SEM ring stage parasite density (per ml blood) for two parasite doses presented as (A) single time series and (B) proportion ring stage parasites presented as an actogram to correct for density differences and visualize change over sequential IDCs and (C) ring stage parasite density presented as an actogram. WT (C57BL/6J) mice in 4 cohorts and housed in LD received parasites from donors housed in DL at a low (1×10^5 parasitized RBCs) or high dose (1×10^7). Shading represents time at which hosts fed (night). Mice (n = 5 infections per cohort for each dose) were sampled every 4 hours for 32h starting at 08:00 GMT

3.4.2 | Disease severity

RBC loss during in the pre-peak window of the infections was best explained by the additive effects of day PI and treatment (ΔAICc = 0, weight =0.96; Table S8; Figure S5a). *Per1/2*-null TRF hosts experienced the greatest RBC loss (mean RBC loss ± SEM × 10^9: 3.29 ± 0.41) followed by WT matched (2.66 ± 0.46) and *Per1/2*-null all-day fed (2.32 ± 0.39) hosts, whilst WT mismatched hosts experienced the least RBC loss (1.48 ± 0.32). Overall, hosts in all treatments lost ~2.0 ± 0.3 (×10^9) RBCs daily across days 2–5 PI with greater loss occurring between days 5–6 PI (mean RBC loss ±SEM × 10^9 = 3.83 ± 0.42). During the post-peak window of the infections RBC loss was best explained by day PI alone (ΔAICc = 0, weight = 0.95; Table S8; Figure S5b) as incorporating treatment reduced model fits (ΔAICc = 5.84, weight = 0.05). Because hosts recovered from anaemia during the post-peak window, RBC switched from a loss of 0.89 ± 0.16 (mean ± SEM × 10^9) on days 7–8 PI to a gain by days 9–10 (−0.56 ± 0.11).

Weight loss during both the pre- and post-peak windows are best explained by day PI alone (pre: ΔAICc = 0, weight = 0.46; post: ΔAICc = 0, weight = 0.78; Table S8; Figure S5c,d). However, AICc model weights in these analyses are low (<50%) indicating high model selection uncertainty. Hosts experienced an average daily weight loss of 0.7 ± 0.08g during the pre-peak window and loss was

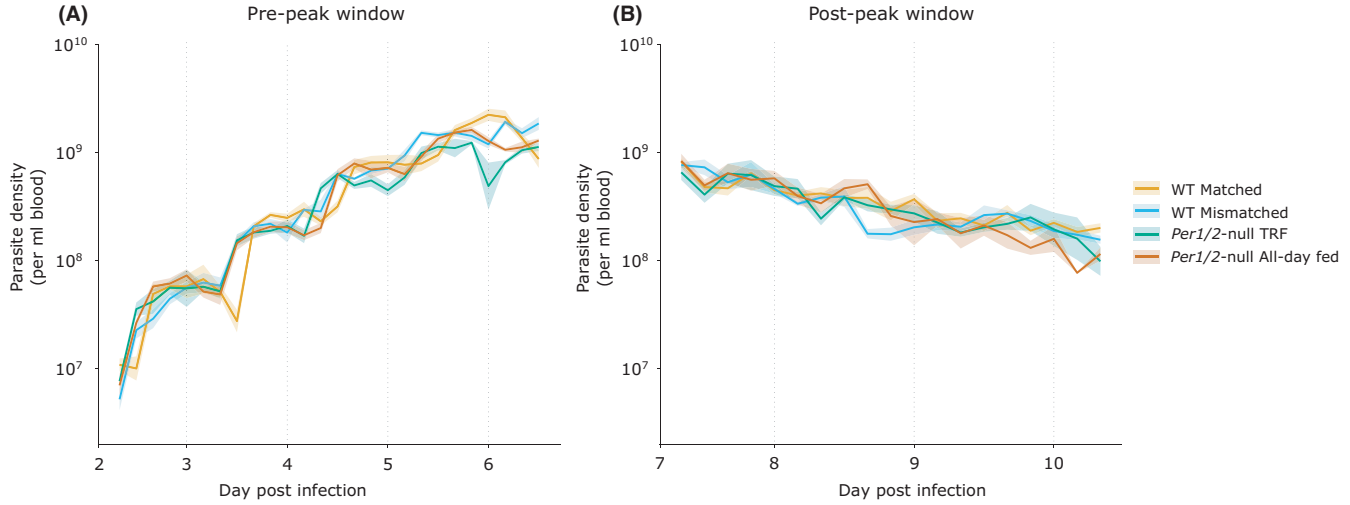

FIGURE 6 Mean ± SEM parasite densities (per ml blood) from concatenating cohorts. Mice were either WT (C57BL/6J) or clock-disrupted *Per1/2*-null mice with parasites that were matched to the host's feeding-fasting rhythm (WT matched), forced to reschedule to align with the host's feeding-fasting rhythm (WT mismatched & *Per1/2*-null TRF) or experienced arrhythmic hosts (*Per1/2*-null all-day fed). (a) n = 4 infections per cohort for all groups in the pre-peak window. (b) For the post-peak window, n = 4 for WT groups, n = 3–4 for *Per1/2*-null TRF, and n = 2–4 for the *Per1/2*-null all-day fed group. Sampling occurred every 4 hours starting at 08:00 GMT during the pre-peak time series (days 2–6 PI) and also for the post-peak time series (days 7–10 PI)

maintained during the post-peak window (weight loss mean ± SEM (g): days 7–8 PI = 1.05 ± 0.31, days 8–9 PI = 0.61 ± 0.0.17) until days 9–10 PI when weight was gained (−0.45 ± 0.16).

4 | DISCUSSION

Here, by analysing ~1200 samples, we demonstrate that phenotypic plasticity in the IDC duration allows *P. chabaudi* to recover from a ~12-hour mismatch to host feeding-fasting rhythms within approximately 5–6 days (Figures 3 and 4), in a manner independent of parasite density (Figure 5). Specifically, by speeding up ('phase advancing') each IDC by 2–3 hours, the timing of peak ring stages shifts within 5–6 IDCs to synchronize with the host's feeding-fasting rhythm (Figure 4b,c). During rescheduling (ie WT mismatched and *Per1/2*-null TRF groups), parasites experience minor reductions in synchrony (Figure 4a) but do not incur costs in terms of the densities achieved during either the pre- or post-peak window of infections (Figure 6), and infecting an arrhythmic host (ie *Per1/2*-null all-day fed) does not impact parasite density (Figure 6).

Whilst our aim was to investigate the ecology surrounding rescheduling of the IDC, we also tested whether this process has longer-term consequences throughout infections. We find that post peak, synchrony degrades in all groups (Figure 4a), the timing of peak ring density shifts or becomes more variable within groups (Figure 4b,c), and IDCs 3–4 became 3–4 hours longer in infections that had to reschedule (ie WT mismatched and *Per1/2*-null TRF groups) and 6 hours longer in *Per1/2*-null all-day fed hosts (Table 1). Whilst the increased variability and dampening of rhythms in the post-peak phase reduces confidence in the precision of period estimates, multiple approaches suggest that infections matched to host rhythms from the outset (WT matched) experienced the least disruption to period, synchrony and timing in the post-peak window. During rescheduling, parasites exhibit altered transcriptional patterns associated with many important processes.[25] Thus, stress experienced during rescheduling may have long-term effects that render parasites more vulnerable to IDC disruption from the stress of host sickness. For hosts, their phase relationship with the parasite's IDC has a minor impact on virulence (Figure S5). Specifically, *Per1/2*-null TRF hosts experience the most severe anaemia, losing approximately twice as many RBC as WT mismatched hosts, with *Per1/2*-null all-day fed and WT matched hosts experiencing an intermediate loss. However, these differences do not extend into the post-peak phase and are not reflected in variation in weight loss, our other virulence measure. This suggests that hosts maintained similar relative levels of food intake across treatments and so, food levels and the impacts of sickness on host rhythms are not the sole drivers of the IDC schedule.

Intuition suggests there are several strategies that parasites could use to reschedule to a new host rhythm, including i) pausing IDC progression for ~12 hours; ii) undertaking an initial large phase shift within the first IDC, followed by fine-tuning the schedule in subsequent IDC; iii) individual parasites within an infection employing different strategies, with some parasites speeding-up and others slowing the IDC; or iv) changing the IDC duration by a fixed amount each cycle (faster or slower) and making linear progress to the correct alignment with host rhythms. Observing a single large shift in IDC timing (as predicted by option (i) or (ii)) could also be due to host rhythms imposing the IDC schedule by severe negative selection of mistimed IDC stages at a certain time of day. In contrast, exposure to a danger at a set time of day could not masquerade as options (iii) or (iv). That we do not observe a severe reduction in densities over a single IDC in WT mismatched and *Per1/2*-null TRF infections, coupled with revealing malaria parasites adopt option (iv) demonstrates that parasites exert more control over their IDC schedule than negative selection by host rhythms. Why would parasites reschedule by changing the IDC duration by a fixed amount each cycle, and why is the period shortened by only 2–3 hours? Extending the IDC by 2–3 hours would align its schedule to host rhythms at the same rate but slowing down development or reducing overall replication rate might render parasites vulnerable to immune killing and delays building a source population for transmission stage production. Similarly, simply waiting for ~12hrs would incur a delay to replication. That we observe a 2–3 hr change in IDC duration is consistent with the recent discovery that loss of serpentine receptor 10 (SR10) causes *P. chabaudi's* IDC to speed up by ~2 hours.[25] Perhaps parasites only express SR10 when in synchrony with host rhythms as a mechanism to maintain this schedule alignment? The IDC changed analogously in both types of rescheduling infection (WT mismatched and *Per1/2*-null TRF); period estimates were similar for both groups and the phase of ring stages shifted to peak at the end of the feeding window, although the phase for WT mismatched parasites was more similar to the WT matched controls (Figure 4c). This could be because parasites can use timing information from additional rhythms operating in WT hosts. Alternatively, it might be optimal for ring stages to peak at the end of the feeding period, and parasites in *Per1/2*-null TRF hosts can achieve this because they are not subjected to the potentially conflicting impacts of other host rhythms present in WT hosts.

Mismatch between the IDC schedule and host rhythms has been reported to reduce asexual replication rate and gametocyte densities during the pre-peak window and also disrupt the expression patterns for genes involved in important cellular processes.[25,28,29,55] Parasites are thought to align to host rhythms to exploit rhythmic resources required from the host's food and to ensure transmission stages mature at the time-of-day mosquitoes seek blood meals.[31] Thus, we expected the costs imposed by resource limitation starving certain mistimed stages plus any role of parasite-parasite communication in rescheduling, being exacerbated at high densities and so, leading to the high dose infections rescheduling sooner. However, across both experiments with infective doses spanning 3 orders of magnitude, all parasites rescheduled via a 2–3 hour reduction in IDC duration and reached the same phase within 5–6 days PI. This suggests that regardless of circumstances, parasites are constrained to reschedule via a set reduction in the IDC duration. Such a strategy could be deployed without parasites needing to communicate, but it remains possible that cell-cell communication[56] is involved and that

signals relating to how much the IDC duration should alter become saturating even at low density. Nonetheless, observing the same reduction in IDC duration across doses suggests this is the minimum duration for the IDC.

In contrast to previous studies,[28,29] we did not detect a reduction in overall asexual densities in mismatched compared with WT matched infections. Thus, why should parasites reschedule if there no apparent costs of mismatch? Whilst experimental designs here—notably sampling regimes and parasite genotypes used—differ with previous studies there are several other non-mutually exclusive explanations. First, altered gene expression pattern of mismatched parasites[25] suggests there are fitness consequences of the IDC schedule. Perhaps by altering cellular processes, parasites can compensate for costs of mismatch in well-fed naïve hosts (as we used in here) because they are able to support parasites at any IDC stage throughout the circadian cycle. This scenario suggests that by establishing the correct IDC schedule early in infection, parasites are anticipating the need to mitigate against future resource limitation that would occur if mismatched and at high density in an ill host. Second, rescheduling must have a (hidden) negative impact on parasites to explain why a faster IDC does not lead to higher overall replication than for the WT matched controls. For instance, if the IDC is reduced by 2 hours, rescheduling parasites complete six IDCs 12 hours ahead of the WT matched parasites, but do not reach higher densities for the same age of infection. Perhaps, parasites trade-off a faster IDC for a reduction in the number of quality of merozoites to maintain the same overall replication dynamics as parasites in control infections. Third, the ultimate selective driver for *P. chabaudi's* IDC schedule might be to coordinate transmission stage maturation with vector biting rhythms, and host-feeding rhythms are a useful proxy for vector rhythms. These ideas could be tested by comparing matched and mismatched infections in hosts with different levels of physiological condition. Fourth, the *P. chabaudi* clone used here (DK) is less virulent than those used in previous studies[57] and so may experience less severe costs of any resource limitation due to misalignment of the IDC.

We examined IDC rhythms in *Per1/2*-null all-day fed infections to establish how the IDC schedule is affected when parasites are neither mismatched nor exposed to time-of-day information. As expected, the IDC rhythm became dampened and its duration reduced, which may suggest a short-free running period (if the IDC schedule is driven by an endogenous oscillator[32,33]). Based on previous studies, we expected synchrony in *Per1/2*-null all-day fed infections to be eroded faster that we observed.[19] Previous experiments followed parasites in singly housed mice, so it is possible that group-housing in the present experiment maintained residual rhythms established by masking during the rearing of mice.[58] Alternatively, other TTFL-independent oscillators may be present in *Per*1/2-null hosts, for instance, food-anticipatory behaviours or non-transcriptional oscillators that influence the IDC schedule.[19] In keeping with a lack of overall costs to rescheduling parasites, infecting an arrhythmic host does not impact on asexual replication. However, exploiting an arrhythmic host might be best achieved by parasites without an IDC rhythm. Future work could examine whether parasites benefit from matching their IDC rhythmicity to the degree of rhythmicity their host exhibits.

Reflecting the lack of overt costs of perturbing the alignment of host and parasite rhythms on asexual density dynamics, we only observe minor differences in virulence between the groups. *Per1/2*-null hosts tend to experience greater anaemia than WT hosts because mice deficient in *Per2* exhibit high susceptibility to acute erythrocyte stressors.[59] However, anaemia dynamics are not related to whether parasites are rescheduling or experiencing dampened rhythms, do not extend into the post-peak window, and weight loss does not vary between treatment groups. Thus, relative to the impacts of infection *per se*, the alignment of host and parasite rhythms appears inconsequential. Hosts experienced more severe symptoms during the post-peak window (eg RBC densities drop to 20% of pre-infection levels) and this likely explains the substantial variation in IDC rhythms in the post-peak window. During the post-peak window, IDC rhythms in all groups experienced substantial reductions in synchrony, variable phase changes and lengthened periods, although the WT matched group was least affected. The impacts of illness on host feeding behaviour coupled with dampened locomotor and temperature rhythms and ~1–3 h advancement of peak timing for these host rhythms[60] may make it difficult for parasites to maintain an IDC schedule.

In summary, our experiments reveal that plasticity in the IDC schedule allows malaria parasites to reschedule following mismatch to host rhythms by reducing the IDC duration by 2–3 hours. This reduction in IDC duration might represent the minimal amount of time required to complete the IDC. The lower and upper limits of IDC duration are unknown but might be revealed by examining parasites in hosts with shorter or longer feeding-fasting cycles. Neither parasites nor hosts experience significant short or long-term consequences of perturbing the alignment between rhythms. However, some costs or trade-offs appear to be involved in rescheduling because a faster IDC does not enhance overall asexual replication dynamics relative to matched parasites. This suggests parasites are able to maintain asexual densities whilst rescheduling, perhaps by trading IDC duration off against merozoite production.[61] Such an ability to compensate might be expected to evolve if parasites often experience circumstances that require rescheduling, such as if egress from the liver is arrhythmic or occurs at a time of day misaligned to feeding-fasting rhythms and highlights the importance of alignment with host rhythms for blood-stages. Furthermore, there may be costs of rescheduling for transmission stage production, although rescheduling parasites do not appear to invest less in transmission.[55] Understanding the extent of, and limits on, plasticity in the IDC schedule may reveal targets novel interventions, such as drugs to disrupt IDC regulation and preventing tolerance to existing drugs by IDC dormancy.

ACKNOWLEDGMENTS

We thank the Reece lab and Giles K. P. Barra for technical assistance and helpful discussions.

AUTHOR CONTRIBUTIONS

AO'D and SR conceived and designed the project. AO'D carried out the experiments. All authors interpreted the data, prepared the manuscript and approved the final manuscript.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/pim.12898.

DATA AVAILABILITY STATEMENT

The datasets supporting the conclusions of this article are available in the Edinburgh DataShare repository: https://doi.org/10.7488/ds/3065.

ORCID

Aidan J. O'Donnell https://orcid.org/0000-0003-3503-094X
Megan A. Greischar https://orcid.org/0000-0002-7521-9344
Sarah E. Reece https://orcid.org/0000-0001-6716-6732

REFERENCES

1. Bloch G, Bar-Shai N, Cytter Y, Green R. Time is honey: circadian clocks of bees and flowers and how their interactions may influence ecological communities. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 2017;372(1734):20160256.
2. Pittendrigh CS. Circadian systems. I. The driving oscillation and its assay in Drosophila pseudoobscura. *Proc Natl Acad Sci*. 1967;58(4):1762-1767.
3. Rymer J, Bauernfeind AL, Brown S, Page TL. Circadian rhythms in the mating behavior of the cockroach, *Leucophaea maderae*. *J Biol Rhythms*. 2007;22(1):43-57.
4. Prior KF, Rijo-Ferreira F, Assis PA, et al. Periodic parasites and daily host rhythms. *Cell Host Microbe*. 2020;27(2):176-187.
5. Westwood ML, O'Donnell AJ, de Bekker C, Lively CM, Zuk M, Reece SE. The evolutionary ecology of circadian rhythms in infection. *Nature Ecol Evol*. 2019;3(4):552-560.
6. Kitchen GB, Cunningham PS, Poolman TM, et al. The clock gene Bmal1 inhibits macrophage motility, phagocytosis, and impairs defense against pneumonia. *Proc Natl Acad Sci*. 2020;117(3):1543-1551.
7. Scheiermann C, Gibbs J, Ince L, Loudon A. Clocking in to immunity. *Nat Rev Immunol*. 2018;18(7):423-437.
8. Hawking F. The 24-hour periodicity of microfilariae: biological mechanisms responsible for its production and control. *Proc R Soc Lond B Biol Sci*. 1967;169(1014):59-76.
9. Rijo-Ferreira F, Pinto-Neves D, Barbosa-Morais NL, Takahashi JS, Figueiredo LM. *Trypanosoma brucei* metabolism is under circadian control. *Nature Microbiology*. 2017;2(6):17032.
10. Hevia MA, Canessa P, Müller-Esparza H, Larrondo LF. A circadian oscillator in the fungus *Botrytis cinerea* regulates virulence when infecting *Arabidopsis thaliana*. *Proc Natl Acad Sci*. 2015;112(28):8744-8749.
11. Lu D-B, Wang T-P, Rudge JW, Donnelly CA, Fang G-R, Webster JP. Evolution in a multi-host parasite: chronobiological circadian rhythm and population genetics of *Schistosoma japonicum* cercariae indicates contrasting definitive host reservoirs by habitat. *Int J Parasitol*. 2009;39(14):1581-1588.
12. Su J, Zhou F, Lu D-B. A circular analysis of chronobiology of *Schistosoma japonicum* cercarial emergence from hilly areas of Anhui, China. *Exp Parasitol*. 2013;135(2):421-425.
13. Martinaud G, Billaudelle M, Moreau J. Circadian variation in shedding of the oocysts of *Isospora turdi* (Apicomplexa) in blackbirds (*Turdus merula*): an adaptative trait against desiccation and ultraviolet radiation. *Int J Parasitol*. 2009;39(6):735-739.
14. Dolnik OV, Metzger BJ, Loonen MJ. Keeping the clock set under the midnight sun: diurnal periodicity and synchrony of avian Isospora parasites cycle in the High Arctic. *Parasitology*. 2011;138(9):1077.
15. Zhuang X, Tsukuda S, Wrensch F, et al. The circadian clock component BMAL1 regulates SARS-CoV-2 entry and replication in lung epithelial cells. *iScience*. 2021;24(10):103144.
16. Zhuang X, Forde D, Tsukuda S, et al. Circadian control of hepatitis B virus replication. *Nat Commun*. 2021;12(1):1-13.
17. Edgar RS, Stangherlin A, Nagy AD, et al. Cell autonomous regulation of herpes and influenza virus infection by the circadian clock. *Proc Natl Acad Sci*. 2016;113(36):10085-10090.
18. Rawlinson KA, Reid AJ, Lu Z, et al. Daily rhythms in the transcriptomes of the human parasite *Schistosoma mansoni*. *bioRxiv*. 2021.
19. O'Donnell AJ, Prior KF, Reece SE. Host circadian clocks do not set the schedule for the within-host replication of malaria parasites. *Proc R Soc B: Biol Sci*. 2020;287:1932, 20200347.
20. Prior KF, van der Veen DR, O'Donnell AJ, et al. Timing of host feeding drives rhythms in parasite replication. *PLoS Pathog*. 2018;14(2):e1006900.
21. Hirako IC, Assis PA, Hojo-Souza NS, et al. Daily Rhyhms of TNFalpha expression and food intake regulate synchrony of plasmodium stages with the host circadian cycle. *Cell Host Microbe*. 2018;23(6):796-808 e6.
22. Mideo N, Reece SE, Smith AL, Metcalf CJ. The Cinderella syndrome: why do malaria-infected cells burst at midnight? *Trends Parasitol*. 2013;29(1):10-16.
23. Garcia CR, Markus RP, Madeira L. Tertian and quartan fevers: temporal regulation in malarial infection. *J Biol Rhythms*. 2001;16(5):436-443.
24. Reece SE, Prior KF. Malaria makes the most of mealtimes. *Cell Host Microbe*. 2018;23(6):695-697.
25. Subudhi AK, O'Donnell AJ, Ramaprasad A, et al. Malaria parasites regulate intra-erythrocytic development duration via serpentine receptor 10 to coordinate with host rhythms. *Nat Commun*. 2020;11(1):2763.
26. Prior KF, Middleton B, Owolabi AT, et al. Synchrony between daily rhythms of malaria parasites and hosts is driven by an essential amino acid. *Wellcome Open Res*. 2021;6(186).
27. O'Donnell AJ, Mideo N, Reece SE. Erratum to: disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated. *Malar J*. 2014;13(1):503.
28. O'Donnell AJ, Mideo N, Reece SE. Disrupting rhythms in *Plasmodium chabaudi*: costs accrue quickly and independently of how infections are initiated. *Malar J*. 2013;12:372.
29. O'Donnell AJ, Schneider P, McWatters HG, Reece SE. Fitness costs of disrupting circadian rhythms in malaria parasites. *Proc Biol Sci*. 2011;278(1717):2429-2436.
30. Owolabi AT, Reece SE, Schneider P. Daily rhythms of both host and parasite affect antimalarial drug efficacy. *Evol Med Public Health*. 2021;9(1):208-219.
31. Schneider P, Rund SSC, Smith NL, Prior KF, O'Donnell AJ, Reece SE. Adaptive periodicity in the infectivity of malaria gametocytes to mosquitoes. *P Roy Soc B-Biol Sci*. 2018;285(1888):20181876.
32. Rijo-Ferreira F, Acosta-Rodriguez VA, Abel JH, et al. The malaria parasite has an intrinsic clock. *Science*. 2020;368(6492):746-753.
33. Smith LM, Motta FC, Chopra G, et al. An intrinsic oscillator drives the blood stage cycle of the malaria parasite *Plasmodium falciparum*. *Science*. 2020;368(6492):754-759.
34. Prior KF, Middleton B, Owolabi AT, et al. Synchrony between daily rhythms of malaria parasites and hosts is driven by an essential amino acid. *bioRxiv*. 2021:2020.08. 24.264689.
35. Teuscher F, Gatton ML, Chen N, Peters J, Kyle DE, Cheng Q. Artemisinin-induced dormancy in *plasmodium falciparum*: duration,

recovery rates, and implications in treatment failure. *J Infect Dis*. 2010;202(9):1362-1368.
36. Babbitt SE, Altenhofen L, Cobbold SA, et al. *Plasmodium falciparum* responds to amino acid starvation by entering into a hibernatory state. *Proc Natl Acad Sci*. 2012;109(47):E3278-E3287.
37. Muñoz-Guzmán F, Caballero V, Larrondo LF. A global search for novel transcription factors impacting the *Neurospora crassa* circadian clock. *G3 Genes Genomes Genetics*. 2021;11(6):jkab100.
38. Johansson M, Staiger D. Time to flower: interplay between photoperiod and the circadian clock. *J Exp Bot*. 2015;66(3):719-730.
39. Takahashi JS. Molecular components of the circadian clock in mammals. *Diabetes Obes Metab*. 2015;17:6-11.
40. Tomioka K, Matsumoto A. A comparative view of insect circadian clock systems. *Cell Mol Life Sci*. 2010;67(9):1397-1406.
41. Edgar RS, Green EW, Zhao Y, et al. Peroxiredoxins are conserved markers of circadian rhythms. *Nature*. 2012;485(7399):459-464.
42. Reece SE, Prior KF, Mideo N. The life and times of parasites: rhythms in strategies for within-host survival and between-host transmission. *J Biol Rhythms*. 2017;32(6):516-533.
43. Taliaferro WH, Taliaferro LG. Alteration in the time of sporulation of *Plasmodium brasilianum* in monkeys by reversal of light and dark. *Am J Epidemiol*. 1934;20(1):50-59.
44. Prior KF. *The evolutionary ecology of circadian rhythms in malaria parasites*. University of Edinburgh; 2018.
45. Mons B, Janse CJ, Boorsma EG, Vanderkaay HJ. Synchronized erythrocytic Schizogony and Gametocytogenesis of *Plasmodium berghei in vivo* and *in vitro*. *Parasitology*. 1985;91(DEC):423-430.
46. Gautret P, Deharo E, Chabaud AG, Ginsburg H, Landau I. *Plasmodium vinckei vinckei*, *P. v. lentum* and *P. yoelii yoelii*: chronobiology of the asexual cycle in the blood. *Parasite*. 1994;1(3):235-239.
47. Boyd G. Experimental modification of the reproductive activity of *Plasmodium cathemerium*. *J Exp Zool*. 1929;54:111-126.
48. Taliaferro LG. Return to normal of the asexual cycle in bird malaria after retardation by low temperatures in vitro. *J Prev Med*. 1928;2:525-541.
49. Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. *Neuron*. 2001;30(2):525-536.
50. Maywood E, Chesham J, Smyllie N, Hastings M. The Tau mutation of casein kinase 1ε sets the period of the mammalian pacemaker via regulation of Period1 or Period2 clock proteins. *J Biol Rhythms*. 2014;29(2):110-118.
51. Jacobs RL. Role of p-aminobenzoic acid in *Plasmodium berghei* infection in the mouse. *Exp Parasitol*. 1964;15:213-225.
52. Dowse HB. Maximum entropy spectral analysis for circadian rhythms: theory, history and practice. *J Circadian Rhythms*. 2013;11(1):1-9.
53. Wu G, Anafi RC, Hughes ME, Kornacker K, Hogenesch JB. MetaCycle: an integrated R package to evaluate periodicity in large scale data. *Bioinformatics*. 2016;32(21):3351-3353.
54. Zielinski T, Moore AM, Troup E, Halliday KJ, Millar AJ. Strengths and limitations of period estimation methods for circadian data. *PLoS One*. 2014;9(5):e96462.
55. Westwood ML, O'Donnell AJ, Schneider P, Albery GF, Prior KF, Reece SE. Testing possible causes of gametocyte reduction in temporally out-of-synch malaria infections. *Malar J*. 2020;19(1):1-10.
56. Brancucci NMB, Gerdt JP, Wang Cheng Qi, et al. Lysophosphatidylcholine regulates sexual stage differentiation in the human malaria parasite *Plasmodium falciparum*. *Cell*. 2017;171(7):1532-1544.e15.
57. Bell AS, Roode JCD, Sim D, Read AF. Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. *Evolution*. 2006;60(7):1358-1371.
58. Mistlberger RE, Skene DJ. Social influences on mammalian circadian rhythms: animal and human studies. *Biol Rev*. 2004;79(3):533-556.
59. Sun QI, Zhao Y, Yang Y, et al. Loss of the clock protein PER2 shortens the erythrocyte life span in mice. *J Biol Chem*. 2017;292(30):12679-12690.
60. Prior KF, O'Donnell AJ, Rund SS, Savill NJ, Van Der Veen DR, Reece SE. Host circadian rhythms are disrupted during malaria infection in parasite genotype-specific manners. *Sci Rep*. 2019;9(1):1-12.
61. Birget PL, Prior KF, Savill NJ, Steer L, Reece SE. Plasticity and genetic variation in traits underpinning asexual replication of the rodent malaria parasite. *Plasmodium Chabaudi*. *Malar J*. 2019;18(1):1-11.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: O'Donnell AJ, Greischar MA, Reece SE. Mistimed malaria parasites re-synchronize with host feeding-fasting rhythms by shortening the duration of intra-erythrocytic development. *Parasite Immunol*. 2021;00e1–14. doi:10.1111/pim.12898